Evaluation of the abundance of tsetse flies and trypanosome infections with a case study in Ethiopia

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Dissertation submitted in the fulfilment of the requirements for the

Degree of Doctor of Philosophy (PhD) in Veterinary Science

2016

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To cite this thesis:

Reta Duguma Abdi (2016). Evaluation of the abundance of tsetse flies and trypanosome infections with a case study in Ethiopia. Thesis submitted in the fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences, Faculty of Veterinary Medicine, Ghent University.

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Dedication

To my parents, to the poor farmers and to their livestock still suffering from trypanosomosis and tsetse fly bites

Turina keessatti killeen millaan adeemti (Oromo proverb)
By presevering the egg walks on legs (English translation)

Acknowledgements

My heartfelt gratitude goes to my supervisors Prof. Dr. Ir. Luc Duchateau, Dr. Vincent Delespaux and Prof. Merga Bekana for their dynamic and steady guidance, counselling and directing me towards productive life for the completion of this work. Words lack power to completely express what you all have meant during this long turbulent journey. Thank you, Prof. Luc, for believing in me when I was helpless, for leading me to the track and for letting me flourish. Luc, besides your supervision on my research work, you have made my life sweeter with your words, your presence, your actions and behaviour. I have enjoyed your true friendship as you have offered me the permission to be myself and to demonstrate my full potential.

The late Prof. Dr. Peter van den Bossche and Prof. Dr. Redgi de Deken: thanks for initiating me in PhD research and for letting me grow. Peter, here I have achieved your best wishes for me, but you are tragically missed so much. Let your soul rest in peace!

My sincere thank goes to Prof. Dr. Guy Caljon for his guidance in the ITM laboratory, for sharing me his deepest expertise on molecular and serological techniques and for introducing me to nanobodies.

Thank you Prof. Dr. Jan van den Abbeele for your constructive comments during the early phase of my PhD work. Lieve, Ko, Victor, Helke, Dries and all ITM staff of Antwerp thank you. Thanks Dr. Klaartje for sharing with me your ideas and your office. Thanks Andries for facilitating things so that I only had to focus on my work. Thanks Xanthippe for your help during arranging the thesis layout.

My gratitude extends to the Examination Board members: Prof. Dr. Pierre Dorny, Prof. Dr. Redgi de Deken, Prof. Dr. Thomas van Leeuwen, Prof. Dr. Frank Pasmans, Dr. Jan Govaere, and Prof. Dr. Guy Caljon for reading, commenting and giving feedback on my PhD thesis. Thanks, some of you helped me during the start-up, laboratory work or finalization of the work.

Dr. Dinka Ayana, Dr. Tefera Yilma, Dr. Yilkal Asfaw, Dr. Hagos Ashenafi, Mr. Hika Waktole, Dr. Bersissa Kumsa, Dr. Fufa Abunna, Dr. Fikru Regassa, Dr. Gezahegn Mamo, Dr. Ashenafi Feyissa, Wro Brehane Wakjira, Wro Melesech Ketema and the rest of my colleagues at Bishoftu College of Veterinary Medicine and Agriculture: thank you indeed for your support at different stages and encouraging me and my lonely family.

Those who supported and encouraged me at different stages of the study were by far too many to list here. Although space is limited in this thesis to list, you have done the unforgettable deeds in my life towards my success. Truly, thank you very much, indeed!

Dr. Moti Y., Mr. Kassahun Eba, and Mr. Kora Tushune of Jimma University and Dr. Thomas, Dr. Bekele, Dr. Lelisa and Mr. Moges of Kaliti tsetse mass rearing factory: thank you.

Dr. Senbeta, Mr. Abebe, Dr. Dereje, Mr. Tesfaye and Mr. Meles, staff members of Bedele tsetse and trypanosome investigation and control center: thank you.

Duguma Abdi (My dad) and Olike Ba'oo (Mam) thank you for letting me grow in joy and taking me to school. I feel sad that you are not there to see my destiny. Let your soul rest in peace. Thank you Garoma, Hirut, Negasse, Demmashe, Hinsene, Dr Olana, Baacuu, Kulani, Teferi, the families of Obbo Wakjira Tefera and Obbo Eseyas Ayana for your moral support along my journey.

Diribe Girma, my wife, your firm endurance in life and your generous inspiration have excited my strength and fueled my dedication to succeed through this long rough journey. You are stimulus for my success and the mastermind of our-shared family. You are with me all the way in the valleys and mountains of my life; you are so special. Let God bless you. Dirroo, thank you!

My son, Nimona, and my daughter, Keeyeron – "Dhiifama! Isin Jaalladha." You guys, you are so sweet.

Finally, I thank Belgium DGDC and VLIR for financial support.

Above all, Thank You my Eternal God. May this thesis be for Your Glory!!

Table of Contents

List of abbreviations

AAT	Animal African Trypanosomosis
AFLP	Amplified fragment length polymorphism
AT	Abundance of tsetse flies
ATP	Adenosine triphosphate
AUC	Area under the ROC curve
BARP	*Brucei* alanine-rich protein
CATT	Card agglutination test for trypanosomosis
cDNA	complementary deoxyribonucleic acid
CFT	Complement fixation test
CNS	Central nervous system
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization
IAEA	International Atomic Energy Agency
FFLB	Fluorescent fragment-length barcoding method
gGAPDH	glycosomal glyceraldehyde 3-phosphate dehydrogenase
HAT	Human African trypanosomiasis
IgM	Immunoglobulin M
IPTG	Isopropyl β-D-1-thiogalactopyranoside for inducing gene expression of rTsal1
IRR	Incidence rate ratio
ITM	Institute of Tropical Medicine
ITS	Internal Transcribed Spacer

kDNA	Kinetoplast deoxyribonucleic acid
KIVI	Kit for In Vitro Isolation
LAMP	Loop-mediated isothermal amplification
MGE-PCR	Mobile genetic element –PCR
NGU	Nguruman trap
Ni-NTA	Nickel ion – nitrilotriacetic acid agarose to purify the histidine-tagged rTsal1
PA	Peasant association
PATTEC	Pan African Tsetse and Trypanosomosis Eradication Campaign
PCR	Polymerase chain reaction
PCV	Packed cell volume
PoT	Prevalence of trypanosomosis
RBCs	Red blood cells
rRNA	ribosomal ribonucleic acid
ROC	Receiver operating characteristic
SRA	Serum resistance-associated protein
TAg-5	Tsetse antigen-5
rTsal 1	recombinant Tsetse salivary gland protein 1
Tsal 2	Tsetse salivary gland protein 2
TSGF-1	Tsetse salivary gland growth factor-1
USD	United States Dollar
VATs	Variable antigenic type
VSGs	Variable surface glycoproteins

Chapter 1:

General overview on the trypanosome–tsetse–host interactions and the epidemiology and control of trypanosomosis and its vector

1. Introduction

African trypanosomosis is a fatal disease of both human (Human African Trypanosomosis (HAT) or sleeping sickness) and livestock (Animal African Trypanosomosis (AAT) or nagana). African trypanosomosis is present in 38 African countries between 15° north and 29° south covering 10 million km² of land and thus putting 60 million humans and 46 million cattle at risk (Murray et al., 1984; Reid et al., 2000). Besides death, it causes substantial economic losses. AAT results in 3 million cattle deaths with direct productive losses of cattle estimated to be 1 to 1.2 billion USD per year (Mattioli et al., 2004) and all total losses reach about 5 billion USD annually (Murray et al., 1984) and drug treatment costs 35 million USD (Geerts and Holmes, 1998). Controlling African trypanosomosis could lead to a significant economic return to these 38 world's poorest countries (Kristjanson et al., 1999) where per capital income is less than 1 USD per day.

This introductory chapter consists of five different sections presenting an overview of trypanosome–tsetse–host interactions and explains the epidemiology and control of the parasite and the vector. The sections are as follows: *(i)* the trypanosome and its interaction with a range of hosts, *(ii)* the tsetse fly and its interaction with the trypanosome and the host, *(iii)* detection/sampling, monitoring and surveillance tools for trypanosomosis and tsetse, *(iv)* distribution, and abundance of trypanosomosis and tsetse, and *(v)* evolution of treatments and control methods against trypanosomosis and vectors.

1.1. The trypanosome and its interaction with a range of hosts

1.1.1. The trypanosome

Based on size, shape, length, presence of a free flagellum, location of the nucleus, size and location of kinetoplast and visibility of undulating membrane, trypanosomes are classified into three groups: trypanozoon (*Trypanosoma brucei* group), nannomonas (*T. congolense* group) and duttonella (*T. vivax* group) (Uilenberg, 1998). Molecular tools have revealed more diversity among trypanosome sub-species along with newly discovered trypanosomes (Adams et al., 2009 & 2010; Hamilton, 2012) which may require further sub-classification. The *T. brucei* group contains *T.b. brucei, T.b. gambiense,T. b. rhodesiense* and *T. brucei* –like. The *T. congolense* group contains T. *congolense* savannah, *T. congolense* forest, *T. congolense* Kilifi, *T. simiae*, *T. simiae Tsavo, T. godfreyi,* and *T. godfreyi* –like. Finally, the *T. vivax* group contains *T. vivax* A, *T. vivax* B, *T. vivax* C – Nyal and *T. vivax* Mozambique (Urakawa et al., 1998; Adams et al., 2009 & 2010) as shown in Figure 1.1. *T. vivax* and *T. congolense* are more diverse in East Africa than in West Africa (Hamilton, 2012).

Microsatellite analysis, amplified fragment length polymorphism (AFLP) or mobile genetic element (MGE)-PCR methods are used for trypanosome genetic diversity analysis. *T. b. brucei* is highly diverse. *T. b. rhodesiense* ranges from showing low to high levels of diversity depending on the geographical region. *T. b. gambiense* shows low levels of diversity within a specific focus, but strains from different geographical foci are very distinct. *T. congolense* (Savannah) have shown high levels of diversity both within a single geographical region as between regions. However, limited information is available on the diversity between different geographically separated populations of *T. congolense* (Savannah) or within the Kilifi and Forest 'clades' of *T. congolense* (Tait et al., 2011; Koffi et al., 2015). This suggests that the main species of African trypanosomes infecting humans and livestock are genotypically diverse. On the other hand, knowledge of the strain diversity of *T. vivax* has been limited due the difficulty of growing *T. vivax* in laboratory rodents or culture (Tait et al., 2011).

Besides genetic diversity, each of the above trypanosome species also have phenotypic diversity with respect to *(i)* drug resistance (although this trait is not inherently linked to the species) , *(ii)* mean time taken for an infected host to become infectious (pre-patent period), *(iii)* virulence (level of parasitemia), *(iv)* pathogenicity (host tissue damage, anemia/PCV or survival time), *(v)* ability and the mean time taken to infect the tsetse, *(vi)* ability and the mean time taken for a

tsetse fly to become infective (Bengaly et al., 2002; Tait et al., 2011). *T. congolense* and *vivax* are regarded as strictly blood parasites whilst *T. brucei* localizes more in tissues and the brain (Murray, 1989).

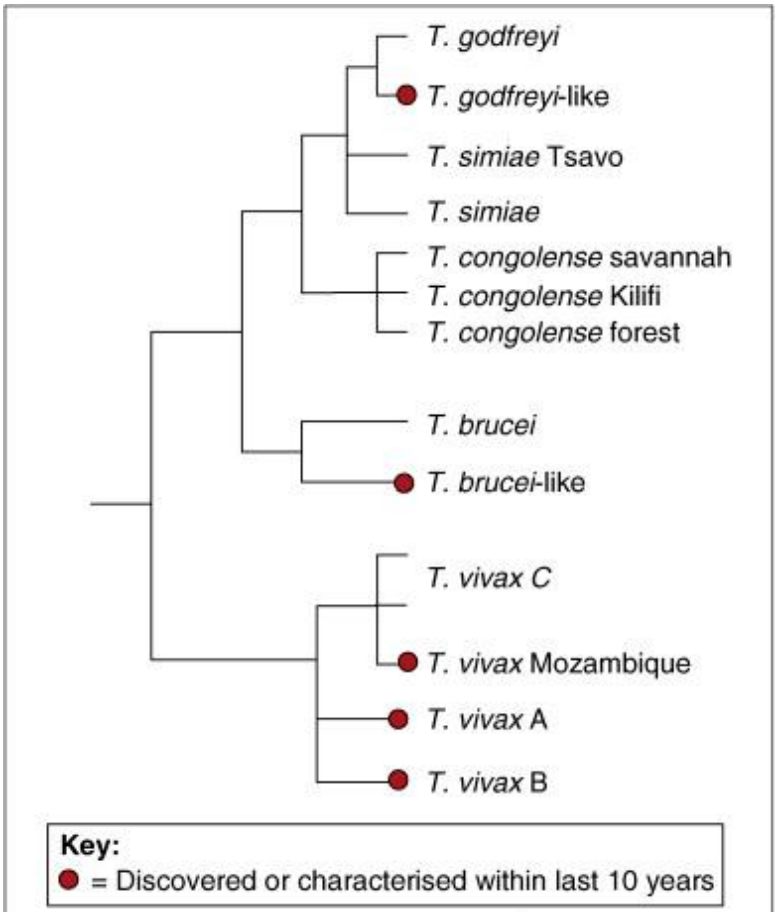


Figure 1.1. Schematic phylogenetic tree on evolutionary relationship among diverse trypanosome species based on combined data from *gGAPDH* and 18S rRNA gene sequences (Urakawa et al., 1998; Adams et al., 2010).

1.1.2. Host range of trypanosomes

A single trypanosome may infect a tsetse fly (Murray, 1989). In turn, one infected tsetse fly may be sufficient to inoculate several hosts (Van Den Abbeele et al., 2010), even up to seven (Jenni et al., 1980). The host range for trypanosomes is diverse due to feeding as the same tsetse flies

might feed on diverse species of mammals (domestic and wild), birds and reptiles as summarised in Table 1.1 below.

Domestic animal such as cattle, sheep, goats, pigs, camels, horses, donkeys, mules, dogs, and cats are affected by African trypanosomosis (Molyneux, 1975) with varying level of tolerance and susceptibility due to parasite and host related factors (Murray, 1989; Bengaly et al., 2002). In general, therapeutic intervention (Van den Bossche et al., 2006), parasite, host, vector, and environmental factors influence the susceptibility, intensity and duration of the disease (Murray, 1989). The rank order for virulence for cattle is *T.vivax* > *T. congolense* > *T. b. brucei*. Trypanosome infection prevalence and virulence varies between host species with cattle > sheep and goats > wildlife. Within one species (e.g. cattle), trypanosome infection prevalence and virulence vary in the order of higher in zebu, moderate in Muturi and lower in N'Dama breeds. Adult animals are also more infected than younger age groups. It also varies due to nutritional status, sex, previous exposure to infection, stress related to reproduction, overwork (ox) and inter-current infection (Murray, 1989).

Wildlife and domestic animals can be sub-clinically infected, chronically sick or fully succumb to the disease depending on predisposing stress factors of the host, host genetic makeup and strain of the parasite. Both wildlife and domestic animals could also serve as a reservoir for a wide range of trypanosome species. Reservoirs are always characterized by low, persisting parasitemia allowing a long or permanent survival of the host remaining infectious to the vector for a prolonged period (Namangala and Odongo, 2014). These reservoirs are source for intra and inter host species transmission of trypanosomes by tsetse flies. Wildlife animals display two categories of response to infection following needle challenge with *T. brucei*, *T. rhodesiense* and *T. congolense.* Species in the first category - gazelle, dik-dik, blue forest duiker, jackal, bat-eared fox, aardvark, hyrax, serval and monkey- usually died of infection. Species in the second category exhibited a spectrum of tolerance ranging from low, moderate and refractory. Some of the less tolerant, such as bushduiker, eland, bohor, reedbuck, hyaena, oribi, bushbuck and impala were susceptible to infection and remained parasitemic for some time. Others with moderate tolerance such as warthog, bushpig and porcupine show very slight and transient parasitemia, whereas baboons were totally refractory to infection. A more limited study found that duiker is resistant to *T. vivax* whereas gazelle is susceptible (Mulla and Rickman, 1988). Furthermore, browser wildlife has higher trypanosome infection prevalence than the open land grazers as browser animals share the preferred environment of tsetse flies. Diurnally active wildlife displays higher infection prevalence than nocturnally active animals as their day-time activity overlaps

with the activity of tsetse fly. Ubiquitously dispersed and abundant wildlife such as warthog has higher infection prevalence than animals with restricted habitat such as hippopotamus and crocodile (Munang'andu et al., 2012).

Wildlife and domestic animals can also serve as a reservoir for trypanosomes causing HAT (Njiokou et al., 2006; Ruiz et al., 2015). A survey was conducted on 1142 wild animals grouped into 36 species to evaluate their reservoir role for *T. b. gambiense* HAT. Of the 36 wildlife species, 20 of them harbour the *T. b. non-gambiense* group and 8 of them the *T. b. gambiense* group (Njiokou et al., 2006).

1.1.3. Trypanosome-host interaction

1.1.3.1. Trypanosome dynamics and virulence in the host

Infected tsetse flies inoculate the metacyclic trypanosome form into the biting site and bloodstream of the host and here the well-studied *T.brucei* is used as a model to explain. In the mammalian host, the metacyclic trypanosome transforms into the typical long slender form. Thus, early in the course of infection the slender form of trypanosomes dominates in the host. The excessive proliferation of the slender parasites (waves of parasitemia) is associated with virulence and pathogenicity (damage to host) and can eventually kill the host. Thus, the parasite needs to devise a strategy to survive itself in the surviving host. In order to regulate and balance the galloping slender form, the quiescent stumpy form is produced. The advantages of producing the stumpy form for the parasite are: *(i)* preventing the uncontrolled proliferation of the slender form that would rapidly kill the host, *(ii)* restraining the confrontation of the slender form with the host, thus, prolonging the within-host infection life-time, *(iii)* the stumpy form is the best form for transmission to the tsetse fly and *(iv)* the stumpy form is more vigorous than the slender to resist enzymatic degradation in the tsetse gut (Matthews et al., 2015).

Table 1.1. Host ranges of trypanosomes in sylvatic and domestic ecosystems

Host species	*T.b.brucei*	*T.b.gambiense*	*T.b.rhodendiens*	*T.congolense*	*T.c.forest*	*T. simiae Tsavo*	*T. simiae*	*T. godfreyi*	*T. vivax*	Any of the trypanosome sp.
Domestic										
Human		+	+							
Cattle	+		+	+			+		+	
Sheep	+		+	+	+		+		+	
Goat	+		+	+	+		+		+	
Camel	+			+					+	
Pig	+			+	+		+		+	
Horse	+			+					+	
Donkey	+			+					+	
Mule										
Dog	+			+	+		+		+	
Primates										
Baboon (*Papio cynocephalus*)				+						
Dwarf guenon (Cercopithecus)		+							+	
Mangabey *(Cercocebus torquatus)*		+							+	
Monkey (*Macaca Fascicularis*)		+			+		+		+	
Vervet monkey (*Cercopithecus* species)										
Potto (Perodicticus potto)		+			+		+		+	
Wild mammals										
Aardvark (*Orycteropus afer)*										+
Bat (*Nycteris* species)										
Bat-eared fox (Otocyon megalotis)										+
Black rhinoceros (*Diceros bicornis*)										
Bohor (Redunca redunca)										+
Buffalo (*Syncerus caffer*)	+			+					+	+
Bushbuck (*Tragelaphus scriptus*)	+			+					+	+
Bushpigs (*Potamochoerus porcus*)										+
Civet cat (*Viverra civetta*)		+		+					+	
Common duicker (*Sylvicapra grimmia*)		+		+	+		+		+	
Dik dik (*Madoqua Kirkii)*										
Duiker (*Sylvicapra grimmia*)										+
Eland (*Taurotragus oryx*)				+					+	+
Elephant (*Loxodonta Africana*)				+						

Table 1.1. Continued

Wild mammals	*T.b.brucei*	*T.b.gambiense*	*T.b.rhodendiens*	*T.congolense*	*T.c.forest*	*T. simiae Tsavo*	*T. simiae*	*T. godfreyi*	*T. vivax*	Any of the trypanosome sp.
Genet (*Genetta genetta*)										
Giraffe (*Giraffa camelopardalis*)	+								+	
Grysbok (*Raphicerus* species)										
Hare (*Hepus capensis*)										
Hartebeest (*Alcelaphus lichtensteini*)	+									
Hyena (*Crocuta crocuta*)	+			+						+
Hippo (*Hippopotamus amphibious*)	+			+					+	+
Hyrax (Hyracoidea)										+
Impala (*Aepyceros melampus*)	+			+						+
Jackal (*Canis mesomelas*)										+
Kudu (*Tragelaphus strepsiceros*)				+					+	+
Leopard (*Panthera pardus*)										+
Lion (*Panthera leo*)	+			+						+
Mongoose (*Herpestes sanguineus*)										
Oribi (Ourebia ourebi)										+
Pangolin (Pholidota)		+							+	
Puku (*Kobus vardoni*)	+			+					+	+
Reedbuck (*Redunca redunca*)									+	+
Roan antelope (*Hippotraggus equinus*)				+					+	
Serval (*Felis serval*)										+
Warthog (*Phacochoerus aethiopicus*)	+			+		+	+	+	+	+
Waterbuck (*Kobus ellipsiprymnus*)	+			+					+	+
Wildebeest (*Connochaetes taurinus*)	+			+					+	+
Wild cats (*Felis lybica*)										
Wild dog (*Lycaon pictus*)	+			+					+	
Zebra (*Equus burchelli*)	+									
Rodents										
Porcupine (*Hystrix galeata*)		+			+		+		+	+
Rat (Rattus)		+			+		+		+	
Squirrel (Sciuridae)		+			+					
Reptiles										
Crocodile (*Crocodylus niloticus*)									+	+
Lizard (Lacertilia)									+	

Modified and compiled from Molyneux (1975), Mulla and Rickman (1988), Njiokou et al. (2004 & 2006), Anderson et al. (2011), Nimpaye et al. (2011), Auty et al. (2012), Munang'andu et al. (2012), Ruiz et al. (2015).

1.1.3.2. Trypanosome host immune system evasion mechanisms

Trypanosomes have developed two systems to evade the host's immune system (Magez et al., 2010; La Greca and Magez, 2011; MacGregor et al., 2012). The first mechanism involves the capability to adjust its own antigen 'appearance'. Four sub-strategies are recognized under this. These include *(i)* varying the structure of their surface coating. Each parasite has a large so-called 'repertoire' of variable antigenic type (VATs) or also called variable surface glycoproteins (VSGs). As the host's immune system responds to one VAT, the parasite switches to another and thereby evades destruction. Within any particular geographical area, there will be several species, subspecies, types and strains of trypanosome, each with their own repertoire of VATs. Consequently, livestock cannot develop effective immunity to the disease (Vickerman, 1978; Baral, 2010; La Greca and Magez, 2011), *(ii)* engulfing VSGs-complexes with antibodies to escape destruction by the host complement, *(iii)* capping or shielding the surface bound immune factors to hide from host immune attack, *(iv)* restricting the invariant receptors in the flagellar pocket, rendering them inaccessible to host immune effectors (Baral, 2010, La Greca and Magez, 2011). The second mechanism relies on weakening the host capacity to mount an efficient response and to maintain its immunological memory (Magez et al., 2010; La Greca and Magez, 2011; MacGregor et al., 2012).

Generally, trypanosomes have the capability to program and change their developmental life cycles both in the mammals and tsetse flies; thus, trypanosomes display an extreme adaptation to their environment. In this regard, they do not only display antigenic variation in their surface coat to evade immune destruction, but also have developed a mechanism to suppress the host immune function through elimination of B and T-cell homeostasis and memory as illustrated in a mouse model. The development of vaccines to prevent trypanosomosis in animals or humans, therefore, has not been successful (Magez et al., 2010; MacGregor et al., 2012).

1.1.3.3. Clinical signs and symptoms of trypanosomosis

In a susceptible host, the clinical signs of disease appear 11-21 days (incubation period) after an infective bite (pathogenic trypanosome) as a relapsing fever. The various species of trypanosome produce various ill effects in different species of livestock. Mixed infections may occur, with corresponding variations in clinical disease (Losos and Ikede, 1972).

The clinical manifestation in animals is influenced by the host as well as the trypanosome species and "strain". In general, local skin reaction (chancre) at bite site, lethargy, weakness, severe

anemia, loss of weight (wasting and emaciation), and intermittent fever are the most commonly seen signs. Hairs are dry and bristling ('staring' of hair coat). After a variable incubation period, fever accompanied by the increase in the number of trypanosomes occurs every 12 days and lasts for 2 to 3 days (Losos and Ikede, 1972). After three to four of these crises (i.e. after about one month), a prolonged period of fever occurs and terminates with the development of severe anemia. The infection may produce peracute, acute, and chronic syndromes. Peracute syndrome is marked by depression, high temperature, lacrimation, photophobia, nasal discharge, and subcutaneous oedema. Chronic trypanosomosis is characterized by weakness, anemia, and emaciation, but the peracute syndromes appear periodically. Other symptoms include reduced productivity, infertility and abortion, with death occurring in some animals during the acute phase of the disease. Animals which survive often remain infected for several months or years, exhibiting a low level of fluctuating parasitemia which serves as a reservoir for the disease. Lacrimation, copious weeping, photophobia to a light, crusting of the discharge at the inner corner of the eyelids and visible swellings of the superficial lymph nodes are more typical of *T. vivax* infections. In general, during parasite destruction by the host antibodies large quantities of trypanosome proteins are liberated into the bloodstream, resulting in a critical situation in the host. Death will commonly occur within one to three months, unless the animal is treated with a trypanocide. The disease is mainly the result of anemia (Losos and Ikede, 1972; Nantulya, 1990).

In humans the chancre (swelling) at the site of the bite, oedema of the face, and anemia are symptoms for *T. b. rhodensiense*. Lymphadenopathy occurs more frequently for *T. b. gambiense*. There are two stages of HAT in both; the early stage refers to the hemolymphatic infection, and the late stage refers to infection of the CNS. The late stage may not occur in West African HAT. However, East African HAT is far more virulent, and can develop into late stage within weeks. Signs associated with nervous system involvement are epileptic attacks; maniacal behaviour, somnolence and coma are some typical late stage symptoms (Dumas and Bisser, 1999).

Most of the clinical symptoms are due to the activity of a set of proteins and enzymes that are released by the parasite in an effort to manipulate and evade the host defence system. The protein families include the famous variable surface glycoproteins (VSGs), glycosyl-inositol-phosphate anchor, cysteine and pyroglutamyl peptidases, inhibitors of cysteine peptidase, oligopeptidases, sialidases, procyclin and apoptotic factor, complement activation factors, trypanosome lymphocyte triggering factors (trypanins), and B-cell mitogens such as proline racemase (Taylor, 1998; Antoine-Moussiaux et al., 2010; La Greca and Magez, 2011).

VSGs are responsible for macrophage over activation resulting in overproduction of harmful substances such as tumor necrosis factor and are also involved in the central nervous system invasion (sleeping sickness), anemia, organ lesions, cachexia and fever (Antoine-Moussiaux et al., 2009; Baral, 2010; La Greca and Magez, 2011). Glycosyl-inositol-phosphate are also involved in the pathogenesis of anemia (Antoine-Moussiaux et al., 2009; Baral, 2010). Cysteine peptidases (congopain) play a role in immune suppression, anemia, thrombocytopenia, central nervous system invasion and regulate excessive parasitemia. Pyroglutamyl peptidases are responsible for total reduction of tyrotrophin-releasing hormone activity and partial reduction of gonadotropin-releasing hormones, thus causing reproduction-related problems in the host (Antoine-Moussiaux et al., 2009; La Greca and Magez, 2011). Oligopeptidases play a key role in endocrine dysfunction and are utilized to degrade several peptide hormones of the host such as neurotensin or atrial natriuretic peptides resulting in hypervolemia (shock) and cardiomyopathy (Antoine-Moussiaux et al., 2009; Baral, 2010). Sialidases play a central role in the alteration of host cell surfaces in red blood cells (RBCs), platelets, leukocytes, and brain cells and subject them to phagocytosis by macrophages resulting in anemia, thrombocytopenia and neurological disorder (sleeping sickness). It also changes the surface of macrophages resulting in loss of its foreign antigen recognition-binding and presentation ability, which results in immune suppression (Antoine-Moussiaux et al., 2009; Baral, 2010). Procyclin and apoptotic factor are involved in the entry of the parasite into the central nervous system via endothelial cell, microglial cell, T and B lymphocyte apoptosis (i.e. trypanosomes cause programmed cell death) with the induction of excessive lesions in the brain, and lymphopenia. Microglial cells are macrophages in the brain (Antoine-Moussiaux et al., 2009; Baral, 2010). Phospholipase is generated by dead trypanosomes. It degrades trypanosome phosphatidylcholine to yield free fatty acids. The free fatty acids cause hemolysis (anemia) and cytolysis in the host (Antoine-Moussiaux et al., 2009; Baral, 2010). Complement-activation factor inhibits the host complement activity, thus rendering the trypanosome resistant to the host complement system attack (Antoine-Moussiaux et al., 2009; Baral, 2010). Trypanosome lymphocyte triggering factor (trypanin) induces over production of interferon-gamma by T cells of the host. Interferon-gamma, in turn, acts as a trypanosome growth factor and stimulates further production of trypanin. Trypanin of the trypanosome collaborates with the over-induced host interferon-gamma for the survival, correct motility, correct trypanosome cell division, multiplication/proliferation, and a prolonged longer-time infection in the host (Baral, 2010; La Greca and Magez, 2011). B-cell mitogens such as proline racemase of trypanosome induce a major immunosuppression as well as “confusion” in

the host. They divert the specific B-cell immune response against the trypanosome towards either to host self-damaging by autoreactive IgM or instruct the B-cell to produce in-effective, non-specific and disordered polyclonal IgM antibodies (Antoine-Moussiaux et al., 2009; Baral, 2010; La Greca and Magez, 2011).

In general, anemia is a cardinal sign and cause of death during trypanosomosis. Pathobiology of trypanosomosis-induced anemia is multifactorial. Mechanical injury of RBCs by the lashing action of powerful locomotory flagella and its micro-tubule-reinforced body of millions of trypanosomes sailing in the bloodstream during parasitemia causes anemia. Undulating fever of the host due to fluctuating parasitemia enhances osmotic fragility and permeability of RBCs also inducing anemia. Protease, neuraminidase, phospholipase, free fatty acids, pyruvates and aromatic by-products are products of live and dead trypanosomes. These toxins and metabolites destroy RBCs. RBCs of infected animals possess decreased anti-oxidant ability. Thus, free radicals and super-oxides from lipid peroxidation can easily destroy RBCs. The RBC cell membrane of infected animals binds readily to antibodies or absorbs trypanosome molecules. Subsequently, such RBCs are destroyed by the antibody-mediated complement system (Mbaya et al., 2012).

1.1.3.4. Host response to trypanosome's exploitation mechanisms

Susceptible animals cannot resist the virulence, pathogenicity and immune evasion tactics of the trypanosomes and thus become sick and eventually die (3 million lethal cases in cattle annually) unless treated. However, trypanotolerant hosts have developed at least two mechanisms to avoid death: *(i)* they control the parasitemia waves (virulence or proliferation) and *(ii)* they limit the pathogenecity (tissue damage) and anemia (Taylor, 1998; Naessens et al., 2002). RBCs of trypanotolerant animals contain more sialic acid and are thus less sensitive to damage by parasite sialidase. They also produce a large amount of antibodies against cysteine protease secreted from trypanosomes, whereas susceptible animals produce a negligible amount. Trypanotolerant animals possess a superior and effective antibody response for neutralisation of parasite factors that prevents the development of pathological features. Trypanosome infected susceptible hosts have a severe depression in complement, but this depression is less pronounced in sera of tolerant cattle breeds, thus, trypanotolerant animals have enhanced complement lysis. Animals confront trypanosomes using their immune system. Disproportionate excessive and ineffective immune response against trypanosomes is common in susceptible hosts which further damages the host's own tissue severely (RBC, spleen, lymph node, myocardium, kidney, liver) whereas

trypanotolerant animals limit their tissue damage. Primates kill most trypanosome species using their trypanolytic factor (Taylor, 1998; Naessens et al., 2002; Antoine-Moussiaux et al., 2009; Baral, 2010; Matthews et al., 2015). Some primates and humans have trypanolytic factors (apolipoprotein L1) in their sera, therefore, most trypanosome species cannot infect humans. However, *T. b. rhodesiense* has developed a serum resistance-associated (SRA) protein whilst *T. b. gambiense* have developed *T. b. gambiense*-specific glycoprotein along with other factors to destroy the host apolipoprotein L1 targeted against the trypanosomes and successfully infect humans, therefore, sleeping sickness is distributed in Africa (Wheeler, 2010; Capewell et al., 2015).

1.2. The tsetse fly and its interaction with the trypanosome and the host

Tsetse flies are taxonomically grouped under the genus *Glossina*, family Glossinidae, and order Diptera. They are categorized into three broad groups based on their habitat preferences, behavioural and morphological characteristics, namely *(i)* the savannah (*morsitans*), *(ii)* the riverine (*palpalis*) and *(iii)* the forest (*fusca*) group (Leak, 1999). These are further divided into 31 species and subspecies. The tsetse fly is a unique hematophagous insect as both male and female feed exclusively on blood (Jordan, 1993; Leak, 1999). Tsetse flies can be easily recognized by their resting posture with the (*i*) wings closed over the abdomen like a closed blades of a pair of scissors (Figure 1.2a), (*ii*) the discal medial cell (dm) or also called hatchet cell of the wing, (*iii*) long piercing proboscis, and the presence of secondary branches on the hairs located on the upper surface of the arista of the antenna (Figure 1.2b) (Jordan, 1993).

Tsetse species are classified into three groups based on external genitalia of both sexes. Females of the *fusca* group have five genital plates, one dorsal pair, one anal pair and a single median sternal plate (Figure 1.3a). Males of the *fusca* group have free superior claspers, without a membrane between them (Figure 1.3b). Females of the *palpalis* group have six genital plates. Besides those present in the *fusca* group, there is one small mediodorsal plate (Figure 1.3c). In males of the *palpalis* group the superior claspers are connected by a thin membrane, deeply divided medially (Figure 1.3d). Females of the *morsitans* group have one pair of fused anal plates (possibly including, medially, the mediodorsal plate of the *palpalis* group) and a single median sternal plate. Besides those present in the *fusca* group, there is one small mediodorsal plate. Dorsal plates are generally absent (Figure 1.3e). In males of *morsitans* group the superior claspers are completely joined by a membrane and are fused distally. Their shape resembles that of mammal scapula (Figure 1.3f) (Jordan, 1993).

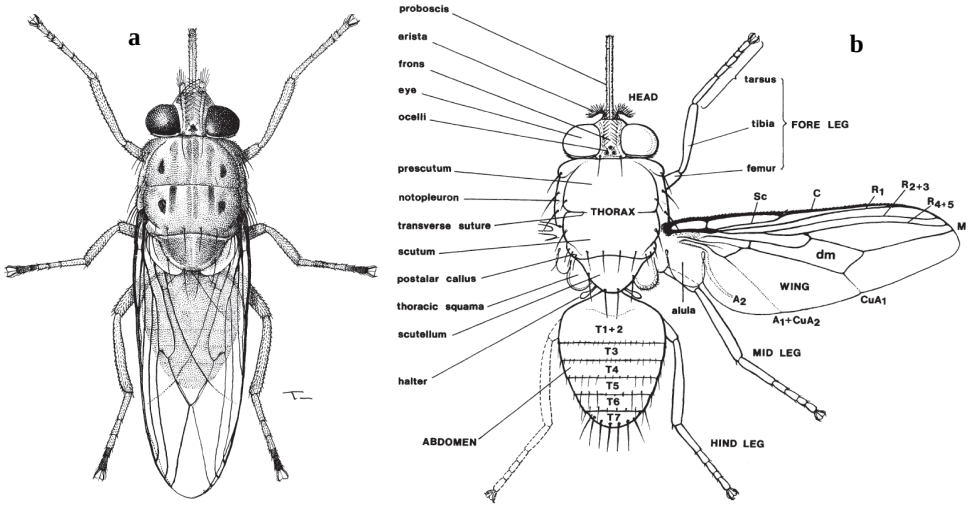
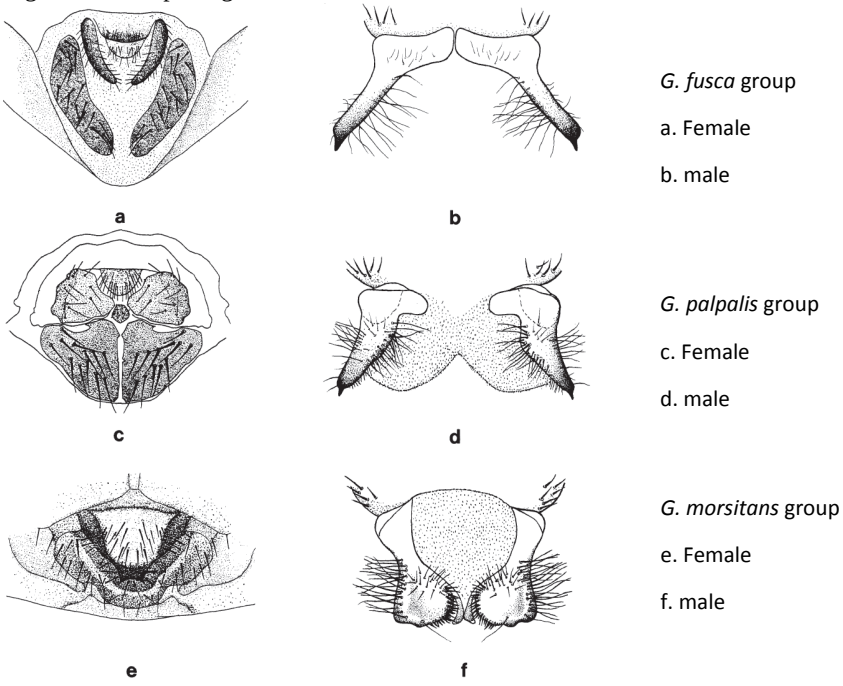


Figure 1.2. Morphological features of tsetse flies.



G. fusca group

a. Female

b. male

G. palpalis group

c. Female

d. male

G. morsitans group

e. Female

f. male

Figure 1.3. Typical forms of female external genital armature and male genital superior claspers in the three species-groups of tsetse fly.

Protein and DNA markers for tsetse fly species differentiation: Traditionally identification of tsetse fly species is based on morphological characteristics, but this approach has limitations. Thus, a number of molecular and genetic markers are developed for differentiating tsetse fly species. The markers are classified into protein and DNA markers. Protein markers are tsetse fly enzymes: either alloenzymes (Gooding, 1992; Krafsur et al., 1997) or isoenzymes. Isoenzymes are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. Isoenzymes markers are used for identification of *G. pallidipes, G. morsitans s.l.* and *G. swynnertoni* (Krafsur and Griffiths, 1992). Another identification method is based on microsatellite markers (at least 20 different microsatellite markers recommended), where the length of a simple and short (2-6bp) repetitive sequence of the DNA is used as a differentiation tool (Luna et al., 2001; Baker and Krafsur, 2001; Krafsur, 2002; Ouma et al., 2003).

Genetic divergence between and within species is present. Large geographical differences within *G. p. palpalis*, suggesting the existence of cryptic species within this subspecies (Dyer et al., 2008).

1.2.1. The behaviour of the tsetse fly

1.2.1.1. The reproduction cycle

Males are sexually mature at five and females at three days. Female tsetse flies mate only once in their lifetime and mostly during their first blood meal right after emergence from the pupal stage. Males mate throughout their entire lifetime. Most female flies are successfully inseminated even at low population densities. Female tsetse flies ovulate (release a single egg) at the age of 7 to 9 days. A single egg is then fertilized by a single spermatozoid (stored in the spermatheca or "sperm bank") which develops into a larva within the uterus, where it is nurtured and supplied with nutrients by a milk gland (Jordan, 1993; Leak, 1999; Hargrove, 2004).

At around the age of 16 days the female tsetse fly releases the first larva. The larva burrows into the ground where it pupates. The pupa stays in the soil for several weeks: the pupal period for males is 28 days and 26 days for females. The inter-larval period (time between the release of larvae) is 9 to 10 days. The female tsetse fly continues to produce a single larva at intervals of 9 to 10 days for her entire lifespan. Unlike other insects, there are no seasonal interruptions (diapause) in the life cycles of tsetse flies. The larvae and pupae, which spend virtually their entire existence either in the uterus or under the ground and are therefore less prone to predation compared to mosquito larvae and pupae that develop in an aquatic environment. However, both

adult longevity and puparial duration are related to temperature, and a significant seasonal decline in tsetse populations is normal, particularly in savannah habitats during the dry season (Jordan, 1993; Leak, 1999).

The increase of tsetse population size is possible if and only if the average female lifespan exceeds 36 days. During optimal conditions, female flies can live as long as 3 months, producing as much as 10 offspring during the lifetime (Jordan, 1986; Jordan, 1993). This life cycle, with a slow reproductive rate of one offspring every 9-10 days and substantial parental investment in the care of young, is a relatively unusual example compared to most insects that produce large quantities of eggs. Tsetse populations are far more vulnerable to disruptions in the life cycle than other insects such as mosquitoes. Thus, control methods such as fly traps, insecticide spraying, and sterile insect techniques have a profound effect on the reproductive rate. This slow rate of reproduction means that tsetse populations can be eradicated by killing just 2-3% of the female population per day (Leak, 1999; Hargrove, 2005).

1.2.1.2. Tsetse population structure: sex ratio, age, life-span and hunger stage

On average, the sex-ratio is about 60-70% with females in excess. However, fluctuation in the sex-ratio is observed as a function of tsetse species, season and location. Tsetse flies have been observed to reach an age of 226 days, but the age varies with tsetse species, season and location. The typical life-span of tsetse flies corresponds to 160 days in the warm rainy season, 110 days in the cold dry season and only 50 days in the hot dry season. The hot dry season kills mainly the young fraction of the tsetse fly population. Another important parameter of the tsetse population is its hunger stage. Both male and female tsetse flies are obligate blood feeders and feed at 2 to 5 days intervals. Resting flies are less hungry than active flies. Males devote each blood meal to the production of fat, an energy store used mainly for flight activity and females accumulate a reserve of fat from about three feeds during each pregnancy cycle. Female tsetse flies assemble and provide all necessary nutrients to the larva in the uterus to survive the long burial-phase of the pupae in the soil. A greater fat content is found in tsetse flies living in areas with an abundant food supply of host animals than in flies living in areas where food is scarce (Challier, 1982; Leak, 1999).

Understanding tsetse population parameters (growth rate, longevity, female reproduction, pupal duration, age structure, dispersal rate and daily flight times) in the field supports the efficiency of the different vector control methods (Hargrove, 2004; Hargrove, 2005; Peck, 2012).

1.2.1.3. Tsetse fly mobility, dispersal rate and daily distance travel

Tsetse flies do not restrict their movement to familiar areas of their habitats, they rather disperse themselves in a random way (Challier, 1982). Tsetse flies move for about 25-30 minutes/day (Bursell and Taylor, 1980), at speeds of about 20 to 24 km/h, giving a flight distance of about 10 km/day (Gibson *et al.*, 1991). The maximum distance of dispersal is 800m per day, but variation is observed among tsetse species and locations (Challier, 1982). Savannah species such as *G. m. morsitans* and *G. pallidipes* can advance their fly front about 25 km in a year (Hargrove, 2000), whereas *G. p. gambiensis* advance 22 km and *G. tachinoides* 25 km along gallery forests as measured in Burkina Faso. In some occasions, long distance along gallery forests is covered in 4 to 5 days (Cuisance et al., 1985). Due to the dispersal of tsetse flies, areas cleared of tsetse rapidly become re-invaded from adjacent infested areas. Indeed, in experiments carried out in Zimbabwe in the 1980s, virtually every fly in a 4 km² block was killed by aerial spraying in one night. Within a day, the catches in the block were back to normal because flies had swarmed back into the block from neighbouring areas. Controlling tsetse over small areas is like trying to make a hole in a lake with a cup (Vale et al., 1984)!

Female tsetse flies displace or move at a greater rate than males. Young flies with poorly developed flight muscles and old flies with damaged wings displace relatively little, and daily flight times can double or halve according to seasonal temperature. When it is hot and dry, tsetse flies retreat into areas of thick vegetation near water; when it is cooler and wetter, they are more evenly distributed in woodlands and thickets (Vale et al., 2015). The reduction of host animals may increase the rate of fly movement; conversely, a large game population will tend to keep flies replete and fly movement will be reduced (Challier, 1982). Generally, ecological behavior of tsetse fly such as tsetse dispersal, diurnal and seasonal movement as well as host preference for blood meal is guided by the domestic and mainly wildlife animals feeding and resting behavior, diurnal activity, ubiquitous /restricted distribution, and seasonal migration (Munang'andu et al., 2012) and tsetse flies adjust themselves accordingly. In addition, habitat size and shape, and the extent to which dense bushes limit space within the habitats affect the daily displacement of tsetse, reducing it by up to 70%. This explains why the savannah group is most mobile, followed by the riverine and forest group (Vale et al., 2015).

1.2.1.4. Tsetse fly diurnal activity and resting

Tsetse flies have a spontaneous “V” shape activity: highly active in the morning and late afternoon, but low around midday (Brady, 1972). The savannah species, such as *G. morsitans*

and *G. pallidipes*, are active mostly for the first two hours and the last two hours of the day (Hargrove and Brady, 1992). Many of the riverine species such as *G. fuscipes* are active in the middle of the day (Crump and Brady, 1979; Mohamed-Ahmed and Odulaja, 1997). There are exceptions to this general scheme: the savannah species *G. longipennis* is most active just after sunset and *G. austeni*, another savannah species, is active in the middle of the day (Kyorku and Brady, 1994). These innate rhythms are directly correlated with light intensity and temperature, indirectly with relative humidity (Mohamed-Ahmed and Odulaja, 1997). Temperatures less than 14°C can suppress the early morning peak of activity. Conversely, high (>32°C) afternoon temperatures, can suppress the afternoon activity. However, tsetse flies need to feed and so when one peak is suppressed then the other compensates. For example, on very hot days, tsetse flies are most active in the early morning (Kappmeier, 2000a) and rest during the rest of the day. For resting, tsetse flies prefer thin (twigs and creepers) or thick (branches and trunks) woody parts of plants. Tsetse flies prefer to rest on the ground or trees as high as <1m, < 3m or up to 5-6m above the ground depending on tsetse species and temperature. Night resting sites of tsetse flies are the leaves and woody parts such as branch, creeper, liana, root, stem, trunk and twig for the savannah groups and gallery forest and thicket for forest group (Challier, 1982).

1.2.1.5. The role of olfaction and vision of tsetse flies in host finding

Tsetse flies use a combination of smell and sight to locate their hosts, and the relative importance of these two means varies between species. The *morsitans* group is very responsive to odour attractants, and catches can be increased fourfold by baiting traps. The catches of the *palpalis* group are increased less as compared to *morsitans* group by odour attractants. Between these two extremes lie species that only respond to certain chemicals (Gibson and Torr, 1999).

Tsetse flies have colour vision. The numbers of tsetse attracted to a target colour increased in the order yellow < green < red < blue. Black is roughly as attractive as blue but, importantly, tsetse land on black in preference to any other colour. The blue colour is therefore widely used to attract tsetse to an object and black to get them to land on it, which is why many targets are blue and black and why we only need to apply the insecticide to the black portion (Green, 1986 & 1988). Important visual attractants include colour (blue and black are best), size (bigger is better) and shape (horizontal oblongs are more attractive than vertical ones). Putting the attractive elements together would give us a large, black horizontal oblong baited with the attractants - to a tsetse it might look and smell like a buffalo - to us it looks like a target (Gibson and Torr, 1999).

Air-borne chemicals that blood-seeking tsetse flies use to locate a host are diverse. Host odour comprises hundreds of different chemicals. The naturally occurring cattle odours and related compounds that attract tsetse flies are acetone, phenol, *o*-cresol, *m*-cresol, *p*-cresol, 2-ethylphenol, 3-ethylphenol, 4-ethylphenol, 2-*n*-propylphenol, 3-*n*-propylphenol, 4-*n*-propylphenol, 2-*i*-propylphenol, 3-*i*-propylphenol, 4-*i*-propylphenol, 2-methoxyphenol, 3-methoxyphenol, and 4-methoxyphenol (Bursell et al., 1988; Torr et al., 1995). The response of tsetse flies to these odours is influenced by typical daily fluctuations in temperature, relative humidity, radiation, wind speed and vegetation type as they modify air speed (Gibson and Torr, 1999). The benefit of baiting traps with other attractants varies between species. For the savannah flies such as *G. morsitans* and *G. pallidipes*, host odours are the most important whereas these are less important for the riverine species (Gibson and Torr, 1999).

1.2.1.6. Tsetse fly behaviour in host locating: ambush or hunt

Both male and female tsetse flies are obligate blood feeders. Tsetse flies have developed two mechanisms for finding their hosts, *(i)* ambush or 'sit and wait' for a host to pass, and *(ii)* hunt/search actively for the host. Most of the flies arriving at a trap or target are probably actively hunting for a host. Flies attracted to moving objects, such as a moving car, man fly-round, ox fly-round or mobile electric net are probably tsetse flies that were sitting and waiting for a host to pass (Vale, 1974a&b; Hargrove, 1991).

Host searching and location by tsetse is thus a key aspect of disease transmission dynamics. Tsetse flies use visual and olfactory characteristics to recognize potential hosts before initiating host-oriented responses. There are a series of behavioural responses involved in the process of take-off, flight and landing on the host to obtain a blood meal. Host-seeking behaviours are influenced by endogenous and exogenous factors. Endogenous factors include the circadian rhythm of activity level, starvation, age, sex and pregnancy status of the fly (Brady, 1972). Exogenous factors include temperature, vapor pressure, visual and olfactory stimuli, and mechanical stimulation (Huyton and Brady, 1975). There are four stages of host-locating behaviour as described by Wilemse and Takken (1994): *(i)* ranging (hunting or searching) of a host in the absence of an external cue, *(ii)* activation/take-off caused by perception of host stimuli, *(iii)* orientation which is upwind anemotaxis in response to complex chemical and visual stimuli directing the insect to the host, and *(iv)* landing on the host. Generally, the tsetse fly will detect an odour plume upwind until it visually recognizes the host. After landing on the host, heat stimulation cause a probing and feeding response. It was found that feeding activity for the

morsitans group was highest during the early morning and the late afternoon due a combination of both external temperature and circadian rhythm (Brady and Crump, 1978).

Tsetse flies must sense host availability, approach, locate, land, probe and feed on the host. The success of this undertaking is also related to host characteristics. These include coat colour of the host (Green, 1986) e.g. tsetse doesn't see effectively zebra (Gibson, 1992) but attracted to blue or black colour (Green, 1986; Vale, 1993a&b), age of the host (Torr and Mangwiro, 2000; Torr et al., 2006), body size and weight of the host (Vale, 1981), defensive behaviour of the host (Torr and Mangwiro, 2000) e.g. impala are more defensive (Vale, 1977b). An ox of 470 kg produces a plume that attracts savannah tsetse from about 90 m, but the range of olfactory detection for large targets with odour bait was 60m (Vale, 1977a). Attraction to the odour from different types of cattle age groups decreases in the order ox > cow > heifer > calf, and oxen were twice as attractive as calves of less than 12 months old (Torr and Mangwiro, 2000; Torr et al., 2006). Inter-species variation in the physical traits, physiology, behaviour, type and quantity of kairomones of different hosts explain partly the variation in the blood meal source of the different tsetse species. The discovery of these host color and kairomones preferences of the tsetse fly has led to a 10- to 1,000-fold improvement in the cost effectiveness of baits (traps and targets) for surveys and control interventions. Baits are now used preferentially to air and ground spraying of insecticides (Vale, 1993a&b).

1.2.2. Trypanosome-tsetse fly interaction

1.2.2.1. Tsetse infection, trypanosome voyage, maturation and its transmissibility

The life cycle of trypanosomes oscillates between the animal and the tsetse fly as indicated in Figure 1.4. The transmission cycle starts when an infected animal is bitten by a susceptible fly. As the fly takes a blood meal from a mammalian host, there are two major forms of *T. brucei* within the blood: the slender (normal) form and the short stumpy (non-replicating) form. Different trypanosome species develop in different fly organs. *T. vivax* develops exclusively in the mouthparts. For *T. brucei* and *T. congolense* prior establishment in the fly midgut is necessary, then, maturation occurs in the salivary glands and the mouthparts, respectively (Roditi and Lehane, 2008; Sharma et al., 2009). However, of both ingested forms, only the stumpy forms of *T. brucei* and *T. congolense* are thought able to survive the attack of potent proteases present in the tsetse saliva and midgut. In the fly, the stumpy bloodstream form transforms into the procyclic form in the midgut (Dyer et al., 2013). At this juncture, the VSG coat that surrounds the trypanosome is shed off completely and is replaced with a midgut protease resistant coat of

glycoproteins known as procyclins (Ruepp et al., 2015). The triggering factor for transformation might be the presence of proteases in the midgut or the cold shock the trypanosome experiences in travelling between an endotherm and an ectotherm.

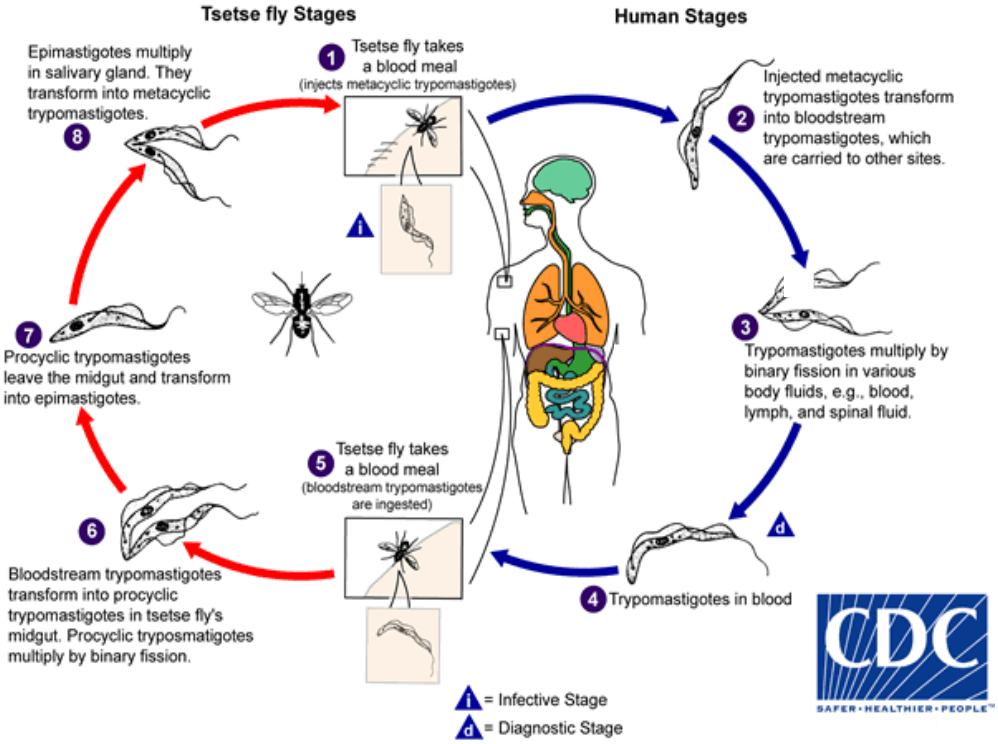


Figure 1.4. The life cycle of the *T. brucei* trypanosome (Geiger et al., 2011; CDC).
During a blood meal on the mammalian host, an infected tsetse fly injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream❶. Inside the host, they transform into bloodstream trypomastigotes ❷, are carried to other sites throughout the body, reach other body fluids (e.g., lymph, spinal fluid), and continue the replication by binary fission❸. African trypanosomes live exclusively extracellularly. The tsetse fly becomes infected with stumpy bloodstream trypomastigotes when taking a blood meal on an infected mammalian host (❹, ❺). In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission❻, leave the midgut, and transform into epimastigotes ❼. The epimastigotes reach the fly's salivary glands, continue multiplication by binary fission and transform to metacyclic stage ❽. The cycle in the fly takes approximately 3 weeks. Humans are the main reservoir for *Trypanosoma brucei gambiense*, but this species can also be found in animals. Wild animals are the main reservoir of *T. b. rhodesiense* (http://www.cdc.gov/parasites/sleepingsickness/biology.html).

Procyclic *T. brucei* expresses the procyclins whilst procyclic and epimastigote *T. congolense* express an immunodominant glutamic acid/alanine-rich protein (GARP) that covers the parasite surface (Hehl et al., 1995). These procyclic forms migrate later to the ectoperitrophic space. In this ectoperitrophic space, they multiply, establish infection and begin to transform into a more motile form, the trypomastigote, that travels upstream along the proventriculus of the fly midgut until it reaches the mouth (*T. congolense*) or salivary gland (*T. brucei*) of the fly (Roditi and Lehane, 2008; Dyer et al., 2013). The trypomastigote changes next into the long epimastigote form by relocating its kinetoplast. The long epimastigote forms asymmetrically divides and transforms in the short epimastigote stage. The epimastigote shields itself from the immune system of the tsetse fly using a surface glycoprotein coat made of brucei alanine-rich protein (BARP). BARP expression is a specific feature of *T. brucei*. Multiple BARP are also used by the epimastigote to promote adhesion to the salivary gland cells. As the fly portion of the trypanosome life cycle comes to a close, the short epimastigotes begin to produce large numbers of metacyclic trypanosomes. The metacyclic trypanosomes are dressed with the VSG coat and procyclin or the BARP cell surface proteins are completely shed (Roditi and Lehane, 2008; Sharma et al., 2009; Dyer et al., 2013).

Besides the transmission of trypanosomes by tsetse flies, biting flies are responsible for mechanical transmission of trypanosomes. A total of 55,398 biting flies were caught by 66 NGU and 20 monoconical traps near lake Tana, Ethiopia, of which 49,353 (89.08%) belong to *Stomoxys*, 4,715 (8.51%) to horse flies and 1,330 (2.4%) to *Chrysops* species. There were no tsetse flies in the traps. Species identification has indicated the presence of *Atylotus agrestis, Chrysops streptobalia, Stomoxys calcitrans, S. nigra, S. pulla, S. pallida, S. sitiens, S. taeniata, S. uruma, Haematopota lasiops* and *Hippobosca variegata*. In the same study, *T. vivax* with prevalence of 6.1% (92/1509) in cattle was recorded, with a single trypanosome species circulating in the area using buffy-coat technique (Sinshaw et al., 2006). In another study, the overall prevalence of trypanosome infections in non-tsetse area was 30.5% with the *T. vivax* prevalence equal to 25.7% (Fikru et al., 2012). The average monthly incidence of trypanosome infection, determined using molecular diagnostic tools, was 20.9% in the non-tsetse areas in which *T. vivax* was responsible for 90.9% of the cattle trypanosome infections (Cherenet et al., 2006).

1.2.2.2. Factors influencing the success of trypanosome infection rates in tsetse flies

All 31 species of tsetse flies can transmit trypanosomes to a greater or lesser extent, but the infection prevalence varies among the tsetse species and decreases with age. However, not all

infectious feeds will produce mature infections in the tsetse fly. Like in the mammalian host, trypanosome's journey, establishment, growth, survival, multiplication, and maturation in the tsetse fly is full of challenges and obstacles (Roditi and Lehane, 2008; Dyer et al., 2013) as shown in Figure 1.5.

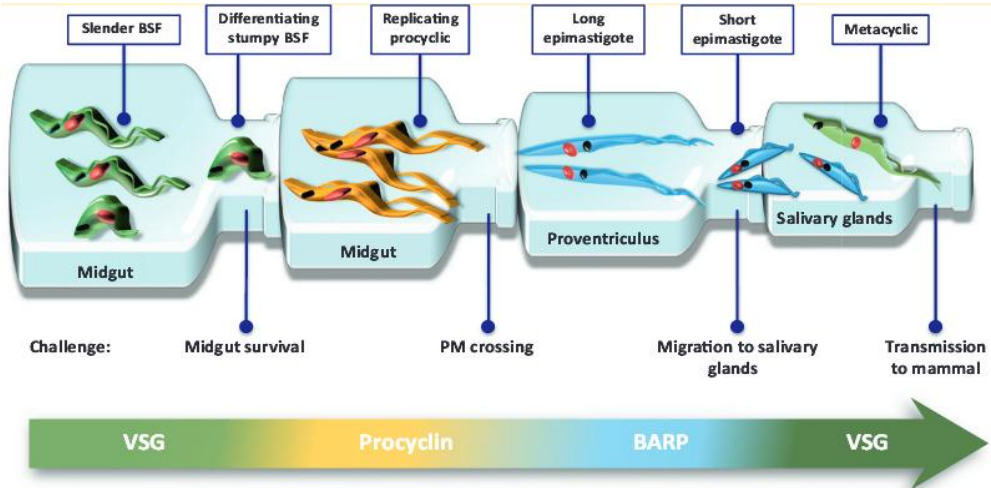


Figure 1.5. Major bottlenecks affecting the transmission of *Trypanosoma brucei* through the tsetse fly (adapted from Dyer et al., 2013).

The endogenous tsetse factors that limit trypanosome infection are tsetse species, age, genetic differences (variations within species), behaviour (host preference), concurrent infections (virus, bacteria, fungi), interactions between lectins and Rickettsia-like organisms and the physiological and biochemical/immune state. Within tsetse species, infection rates vary greatly depending on individual tsetse factors, with dose-related tsetse fly immune proteins. Tsetse fly innate and immune proteins such as lectin, cecropin, attacin, defensin, and diptericin, reactive oxygens (radicals), reactive nitrogens, nitric oxide and hydrogen peroxide destroy the trypanosomes and prevent its establishment in tsetse midgut (Hao et al., 2001; Dyer et al., 2013). More than 20 bacterial floras exist in tsetse gut. Most of them are beneficial for the tsetse fly synthesizing and providing B vitamin for the tsetse fly. Of these bacteria, *Wigglesworthia* also improves tsetse's reproductive fertility, immunity and protects tsetse against viral and trypanosome infections (Pais et al., 2008; Soumana et al., 2013). However, *Wolbachia* infection of tsetse causes reproductive abnormality (particularly embryonic death) in tsetse fly due to disruptions in early fertilization events (Cheng et al., 2000; Alam et al., 2011). *Wolbachia* infections can be detected in 100% of sampled individuals (Cheng et al., 2000), while infections vary significantly in field populations

from 6.7 to 100% (Symula et al., 2013). R*ickettsia*-like organisms facilitate the trypanosome infection of the tsetse fly. The presence of intracellular *rickettsia*-like organisms, also called *Sodalis glossinidius*, in the tsetse fly midgut increases the risk of infection with trypanosomes six fold (Maudlin et al., 1990). Elimination of *Sodalis glossinidius* by streptozotocin treatment results in a 40% reduction in susceptibility to midgut infection in the progeny of treated flies (Dale and Welburn, 2001). *S. glossinidius* is potentially an efficient target for controlling tsetse fly vectorial competence and consequently African trypanosomosis (Farikou et al., 2010a). Chitinase production by the *Sodalis glossinidius* is responsible for chitin degradation, thereby generating carbohydrates that might interfere with immune factors of the tsetse fly, particularly lectins. This ultimately increases tsetse susceptibility to trypanosome infection (Welburn et al., 1993). The parasite factors that determine tsetse infection are the parasite load, parasite species/ subspecies/strain and its infectivity to tsetse (Jordan, 1974; Molyneux, 1980). Infection rates also vary greatly among trypanosome species, with *T. vivax* having the highest prevalence followed by *T. congolense* and *T. brucei* (Molyneux, 1980). This could be related to the life cycle of trypanosome species in tsetse as *T. vivax* has a life cycle of 10 days, *T. congolense* 14 days and *T. brucei* 30 days in tsetse fly (Van den Bossche et al., 2011). Ecological (environment) factors such as climatic factors, hosts available for subsequent feed and availability of infected hosts also determine tsetse infection rate. Trypanosome infection rate in the tsetse fly is also influenced by host factors such as host susceptibility, immune state of host, behaviour and attractiveness of the host to tsetse (Jordan, 1974; Molyneux, 1980).

Flies within the *morsitans* group are highly competent vectors, followed by the *palpalis* group whereas those within the *fusca* group show refractoriness to trypanosome infection. The genetic basis of vector competence in tsetse is largely unknown. (Moloo and Kutuza, 1988a, 1988b). Trypanosome infections have a negative effect on the tsetse fly as it increases its mortality (Woolhouse et al., 1993), and reduces tsetse fly fecundity by 30% (Hu et al., 2008).

1.2.3. Host – tsetse fly interactions

1.2.3.1. Host preference of tsetse for blood meal source

Tsetse flies feed exclusively on blood but blood from particular animals seems to be preferred for some reasons such as host odor. Feeding habits of tsetse flies are determined by blood meal analysis and are divided into five main patterns (Weitz, 1963; Challier, 1982; Moloo, 1993; Clausen et al. 1998) including *(i)* flies feeding mainly on suids such as *G. swynnertoni*, but also

on bovids and carnivores, *(ii)* flies feeding mainly on suids and bovids such as *G. m. morsitans* (60% from suids such as warthogs, bushpigs and 16% from bovids). The blood meal of *G. m. centralis* and *G. m. submorsitans* constitute for 86% of donkey blood, *(iii)* flies feeding mainly on bovids such as *G. pallidipes*, *(iv)* flies feeding mainly on other mammals than suids and bovids such as *G. brevipalpis* feed on bush-buck and hippopotamus, *(v)* flies feeding on the most available host and man. Over 50% of the blood meal of *G. p. gambiensis* comes from reptiles**,** the rest from man and bovids**.** Bush-bucks, oxes and buffaloes are the main hosts for *G. f. fuscipes* but it also feeds on the monitor lizard**.** Bovids, mainly, the bush-buck provide over 65% of the blood meals for *G. tachinoides*, the rest being primates, wart-hogs and reptiles. In this tsetse group reptiles constitute the greatest part of the tsetse fly diet during the cold dry season (Weitz, 1963; Challier, 1982; Clausen et al. 1998) as shown in Table 1.2. Table 1.1 summarizes the different wild and domestic animal species with trypanosome infections, which therefore also serve as bloodmeal source for tsetse flies and thus acquire the trypanosome infection.

The above classification gives a rank order of preference. However, removal of the most favoured hosts from the habitat of the tsetse flies results in adaptation to other available hosts. In this regard, where humans and their livestock are present, many of the natural hosts disappear but domestic livestock such as cattle and pigs act as ideal substitutes for these wild hosts (Weitz, 1963; Challier, 1982; Moloo, 1993; Clausen et al., 1998). In general, the host preference of tsetse fly is affected by several factors: availability of the host, tolerance and defensive behaviour of the hosts, digestibility of the blood of host by the tsetse fly (Weitz and Glasgow, 1956). Knowing the host preference of the tsetse fly has a number of merits: *(i)* identification of reservoir hosts for trypanosomes, *(ii)* elimination of such hosts starves tsetse to death (Weitz and Glasgow, 1956), *(iii)* exploring the mechanisms underlying the tsetse attraction or repletion prompts discovery of new attractants or repellents (Vale, 1993).

1.2.3.2. The role of tsetse fly saliva in tsetse feeding and trypanosome transmission

Tsetse flies rely on their complex salivary protein to inhibit host haemostatic reactions ensuring an efficient feeding. Salivary glands of tsetse flies produce over 250 proteins (Alves-Silva et al., 2010). The four proteins that are the most abundant soluble gene products present in salivary glands of teneral *G. m. morsitans* are: *(i)* tsetse salivary gland growth factor-1 (TSGF-1), *(ii)* tsetse salivary gland protein 1 (Tsal 1), *(iii)* tsetse salivary gland protein 2 (Tsal 2), and *(iv)* Tsetse antigen-5 (TAg-5), which is a member of a large family of anti-haemostatic proteins (Haddow et al., 2002). The tsetse fly salivary gland proteins have an antithrombin anti-coagulant

activity, platelet anti-aggregation activity and thrombin inhibitor activity. They also cause immediate and delayed cutaneous hypersensitivity (Haddow et al., 2002; Caljon et al., 2006; Alves-Silva et al., 2010; Van Den Abbeele et al., 2010).

Besides producing proteins as anticoagulants, the salivary glands of tsetse flies produce molecules that may play both a direct and indirect role in the growth, maturation and transmission of trypanosomes. In this regard, homogenized salivary gland tissue has been shown to initiate the transformation *in vitro* of procyclic (midgut form) trypanosomes into VSG-expressing metacyclic salivary forms that are infective for mice (Cunningham and Honigberg, 1977; Cunningham and Taylor, 1979). In this regard, tsetse fly saliva accelerates the onset of a *Trypanosoma* infection (Caljon et al. 2006). A trypanosome infected tsetse fly produces a lower dose of salivary proteins and its composition is also altered, whereby the tsetse fly's capacity to inhibit host haemostatic reactions is hampered. It therefore has to bite several hosts to feed satisfactorily (Jenni et al., 1980; Van Den Abbeele et al., 2010) which could lead to an increase of the vector/host contact and subsequently more parasite transmission in field conditions.

1.3. Detection, monitoring and surveillance tools of trypanosomosis and tsetse flies

1.3.1. Detection, monitoring and surveillance tools of trypanosomosis

The tools available for diagnosis, monitoring and surveillance of African trypanosomosis can be either based on the presence of clinical symptoms of the host or on detecting the parasite in a direct or indirect way (antibody/antigen detection and DNA detection) (Molyneux, 1975; Nantulya, 1990).

Apart from clinical signs typical for trypanosomosis, the most important clinical parameter is the packed cell volume (PCV). Using a cut-off value of 26%, PCV has a sensitivity of 76% and specificity of 94% to detect cattle trypanosomosis in African endemic areas (Marcotty et al., 2008).

Table 1.2. Host diversity for tsetse species blood source (n = 38,945). Source: [1]Weitz (1963), [2]Clausen et al. (1998). n = total number of blood meal fed tsetse in the field as classified by animal species for blood source.

	swyn [1]	aust [1,2]	fuscipl [1,2]	tabanif [1]	morsit [1,2]	orient [1]	submors [1]	pallid [1]	longipalp [1,2]	fusca [1,2]	longipen [1,2]	brevipalp [1,2]	fuscipes [1,2]	palpalis [1,2]	tachino [1,2]
1. PRIMATES	**211**	**230**	**4**	**0**	**392**	**158**	**247**	**73**	**19**	**0**	**5**	**8**	**107**	**142**	**181**
Man	162	180	2	0	305	96	198	62	14	0	4	7	99	105	126
Baboon	9	9	1	0	14	17	11	0	0	0	0	0	1	2	5
Monkey	6	6	0	0	3	5	11	0	2	0	0	0	1	10	0
Total spp. ID	177	195	3	0	322	118	220	62	16	0	4	7	101	117	131
Total spp. non ID	34	35	1	0	70	40	27	11	3	0	1	1	6	25	50
2. SUIDS	**3617**	**3842**	**657**	**158**	**5424**	**855**	**612**	**803**	**75**	**105**	**516**	**485**	**218**	**707**	**24**
Bushpig	52	240	448	0	902	77	7	301	4	0	168	427	19	30	0
domestic pig	0	0	0	0	15	0	0	0	11	2	0	0	23	283	0
Giant forest hog	0		0	90	0	0	0	0	0	0	0	0	0	0	0
Red river hog	0	0	0	158	0	0	0	4	33	89	0	0	0	8	0
Warthog	3179	3179	6	0	3556	487	493	430	1	1	54	10	11	13	3
wild suidae		0	0	0	186	0	0	0	4	0	51	1	0	0	2
Total spp. ID	3231	3419	544	158	4659	564	500	735	53	92	273	438	53	334	5
Total spp. non ID	386	423	113	0	765	291	112	68	22	13	243	47	165	373	19
3. BOVIDS	**1260**	**1403**	**117**	**24**	**1756**	**974**	**296**	**1705**	**1015**	**520**	**264**	**271**	**223**	**112**	**292**
Buffalo	386	390	17	1	477	39	53	180	142	3	262	123	12	6	18
Bushbuck	5	9	12	10	335	25	51	1099	758	522	1	87	135	187	431
cattle	21	77	115	8	755	82	15	35	19	4	36	15	111	28	38
Duiker	9	38	0	0	108	8	5	2	25	8	4	2	3	8	32
Eland	67	68	0	0	77	46	3	4	0	0	0	2	0	0	0
Gazelles	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0
Giraffe	417	417	0	0	46	15	4	10	0	0	14	1	0	0	0
Hartebeest	9	9	0	0	0	0	21	1	0	0	0	0	0	0	0
Impala	25	25	0	0	10	2	1	0	0	0	0	0	0	0	0
Kob	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Kudu	2	8	0	0	139	413	5	9	0	0	0	0	0	0	0
Oribi	0	0	0	0	0	0	12	0	0	0	0	0	0	1	0
Reedbuck	1	1	0	0	81	15	1	10	0	0	0	0	0	0	0
Roan antelope	34	34	0	0	35	3	38	18	0	0	0	1	0	1	0
Sheep/Goat	19	19	1	0	8	1	3	3	0	0	3	0	0	0	15
Steinbok	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Waterbuck	1	1	0	0	51	1	0	1	1	0	0	8	4	2	38
wild ruminant	0	0	14	0	528	0	0	0	40	24	55	5	156	70	206
Total spp. ID	999	1099	76	19	1208	650	212	1373	816	463	238	220	123	56	41
Total spp. non ID	261	304	41	5	548	324	84	332	199	57	26	51	100	56	251

Table 1.2. Continued

	swyn [1]	aust [1,2]	fuscipl [1,2]	tabanif [1]	morsit [1,2]	orient [1]	submors [1]	pallid [1]	longipalp [1,2]	fusca [1,2]	longipen [1,2]	brevipalp [1,2]	fuscipes [1,2]	palpalis [1,2]	tachino [1,2]
4. OTHER MAMMALS	**370**	**377**	**111**	**52**	**1732**	**357**	**110**	**94**	**35**	**96**	**1173**	**734**	**115**	**125**	**1401**
Aardvark	0	0	0	0	5	6	2	0	1	55	5	0	0	0	0
Cats	6	6	0	0	6	7	11	0	0	0	9	0	0	0	1
Dogs	4	4	0	0	17	4	14	0	0	0	1	0	0	0	0
Elephant	45	46	0	0	252	219	2	28	2	1	181	61	3	1	1
Hippopotamus	0	0	82	0	1251	0	0	14	5	0	70	610	11	14	1286
Hyaena	6	6	0	0	2	0	0	0	0	0	1	1	0	0	0
Other carnivores	17	17	0	0	4	4	4	3	0	0	1	3	16	2	2
Porcupine	0	1	0	50	14	9	21	2	1	8	0	2	0	0	17
Rhinoceros	140	140	0	0	43	46	20	13	0	0	816	18	0	0	0
Other mammals	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Camelidae	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
Canidae	0	0	4	0	13	0	0	0	2	4	32	1	39	25	18
Equidae	0	0	0	0	6	0	0	0	0	0	0	0	0	1	8
felidae (exc lion)	0	0	19	0	15	0	0	0	1	1	13	1	12	55	0
Lion	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rodents	0	0	2	0	3	0	0	0	3	0	4	0	20	14	20
Total spp. ID	218	220	107	50	1631	295	74	60	15	69	1136	697	101	112	1353
Total spp. non ID	152	157	4	2	101	62	36	34	20	27	37	37	14	13	48
5. BIRDS	**64**	**64**	**1**		**33**	**23**	**69**	**7**	**2**	**1**	**129**	**2**	**0**	**51**	**14**
6. REPTILES	**9**	**0**	**0**	**0**	**23**	**1**	**8**	**5**	**30**	**4**	**0**	**0**	**750**	**265**	**401**
crocodile	0	0	0	0	3	0	0	0	1	0	0	0	20	13	29
monizer lizard	0	0	0	0	10	0	0	0	26	0	0	0	527	151	337
n.s. reptiles	9	0	0	0	10	1	8	5	3	4	0	0	203	101	35
Total host exam	**5531**	**5916**	**890**	**234**	**9360**	**2368**	**1342**	**2687**	**1176**	**726**	**2087**	**1500**	**1413**	**1402**	**2313**

Direct parasite detection uses wet blood films, fresh preparation of lymph, thick blood film, and thin blood or lymph smear (Molyneux, 1975; Nantulya, 1990). Parasites can be detected at concentrations of > 10^5 per ml (Murray, 1989). The Woo test (microhaematocrit or capillary tube centrifugation technique) is another parasite detection method (Molyneux, 1975; Nantulya, 1990). In this method, the anticoagulant coated capillary tube is filled with 50 µl blood, centrifuged at high speed (12000g for 5 min) and the presence of trypanosomes is examined in the buffy coat. The Woo test has a sensitivity of 56% (Bonnet et al., 2015). It detects parasites at concentrations of 10^2 to 10^3 per ml (Murray, 1989). During the chronic stage or in trypanotolerant animals, parasites appear in such low numbers and thus need to be concentrated. The mini anion exchange centrifugation technique (column chromatography) is another parasite concentration method (Molyneux, 1975; Nantulya, 1990). Patient's blood is negatively charged and trypanosomes remain neutral and are thus separated by anion exchange chromatography at pH 8. The blood is put on a column containing diethylaminoethyl cellulose; the blood cells stay on the gel and the trypanosomes pass in the column. It has a sensitivity of 77% (Bonnet et al., 2015). Dissection of tsetse flies and microscopy of organs is also used since a long time (Lloyd and Johnson, 1924). An alternative for parasite detection with higher sensitivity is the inoculation of blood, lymph, cerebrospinal fluid or dissolved organ contents of tsetse flies in rats or mice for xenodiagnosis. Its added merit is that field isolates from mammals or tsetse flies can be collected via rodent inoculation for further studies. Its disadvantage is that diagnosis is not immediate, and that *T. vivax* and *T. simiae* do not infect rodents (Molyneux, 1975; Nantulya, 1990; Gibson et al., 1999). Tissue culture technique (in vitro cultivation) can be another option using different culture media, such as Cunningham liquid media (Cunningham and Honigberg, 1977) and Kit for In Vitro Isolation, KIVI (Aerts et al., 1992). Bloodstream forms readily transform to procylin tsetse fly (midgut) forms on transfer from 37°C to a lower temperature incubation of 25 to 28°C (Cunningham and Taylor, 1979). Both bloodstream and procylin forms can be cultured with the procyclin form having a better trypanosome harvest of 10^7/ml than the bloodstream form with 10^5/ml. The culture method is vital as it can provide information on pathogen viability and susceptibility to drugs (Gibson et al., 1999).

In 1899, sera from trypanosome-infected animals caused lysis of trypanosomes, later shown to be both complement- and antibody-dependent. This was the basis for the development of the complement fixation test (CFT) for the diagnosis of trypanosomosis. Currently, CFT is used widely for *T.evansi* and *T. equiperdum* which are non-tsetse transmitted trypanosomes (Nantulya, 1990). Antibody detection using the card agglutination test for trypanosomosis (CATT) has a

sensitivity of 91% and a specificity of 97% when using human blood from a finger prick and is widely employed for HAT screening of human population for *T.g. gambiense* infection. Parasite detection in the lymph node is done for CATT positive humans. It has a sensitivity of 40-80% (Bonnet et al., 2015). ELISA techniques can be applied on a large set of samples but it does not differentiate between present and past infections. Moreover, trypanosome species cannot be discriminated with these serological-based detection methods (Bengaly et al., 1995).

Trypanosome species are differentiated using the species specific isoenzyme patterns. Isoenzyme band patterns of 10-20 different enzyme extracts from the trypanosome cytoplasm are separated on electrophoresis based on their electrophoretic mobility and stained to be visualized. It needs highly concentrated proteins extracts, thus, a minimum of 100 million trypanosomes are needed (Gashumba et al., 1988; Godfrey et al., 1990; Gibson et al., 1999). Since the metabolism of insect and bloodstream forms is different, the isoenzyme bands seen may be different in number, mobility or intensity depending on the life cycle stage used (Gibson et al., 1999).

The first DNA based methods were DNA sequencing and synthesis of DNA-probes, followed by PCR, and a combination of both techniques. DNA probing entails exposing a denatured DNA sample fixed on nitro-cellulose to a labeled DNA-probe under specific salt and temperature conditions. If the complementary DNA sequence is present in the sample, the probes will bind to it and remain on the nitro-cellulose where they can be visualized (with substrate or film). PCR was originally developed to amplify sequences of interest and to increase the sensitivity of detection using DNA-probes. However, since the DNA-probe technique is laborious and time consuming, the development of PCR alone has been, in recent years, mainly devoted to diagnosis without the need of probes (Gibson et al., 1999; Desquesnes and Davila, 2002).

In 1989, the PCR technique exploiting a thermostable enzyme, *Taq* polymerase, which synthesizes a new strand of DNA by copying an original DNA template, was developed for trypanosome identification. The preferred targets are those which are present in a large number of copies in the genome of trypanosomes; the more copies of the target, the greater the chance of amplifying it by PCR. Single copy genes are more difficult to amplify and are rarely targeted since low parasitemia is a characteristic of trypanosome infection in chronic or trypanotolerant animals and the sensitivity would be too low. Subsequently, mini-chromosomes of the nuclear DNA containing satellite DNA, mini-exon genes of the nuclear DNA, kinetoplastid mini-circle DNA sequences are targets in the development of species-specific primers for PCR (Desquesnes and Davila, 2002). To date, a number of loci have been used for intra-specific discrimination of

Trypanosoma species. Among these are the mini-exon or spliced leader, ITS rDNA, microsatellites, mini-satellites, 24S-rDNA, flagellar pocket glycoprotein and kDNA. The common characteristic of these loci is that the amplified DNA segment needs to be variable in either size or sequence, or both. The level of intra-specific polymorphisms varies according to each target and its rate of evolution (Desquesnes and Davila, 2002). PCR has a limitation of *(i)* processing samples post PCR, *(ii)* carry over contamination of PCR products and *(iii)* do not quantify and monitor the amplicon accumulation over time. Real-time quantitative PCR is evolved for trypanosome DNA detection and overcomes these limitations, because post-PCR end point analysis is not necessary. This method is now developed for African trypanosomosis of humans (Becker et al., 2004) and animals (Silbermayr et al., 2013), but is quite expensive and therefore not often used as a diagnostic method for the detection of endemic trypanosomosis in African countries. The loop-mediated isothermal amplification (LAMP) reaction method is evolved to overcome the cost of DNA detection methods such as PCR and real-time PCR. LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions and it is cheap demanding only simple incubators. An added advantage of LAMP over PCR-based methods is that DNA amplification can be monitored spectrophotometrically and/or with the naked eye without the use of dyes (Kuboki et al, 2003). The capacity of conventional PCR (56.25%) for detecting *T. vivax* positive samples was lower than that of LAMP (93.73%). This may influence the choice of screening tests for cattle herds infected with *T. vivax* (Cadioli et al., 2015).

1.3.2. Sampling, monitoring and surveillance tools of tsetse flies

Traps are the most widely used sampling method for surveying, monitoring and surveillance of tsetse fly populations. Several traps are currently available for tsetse sampling as shown in Figure 1.6. Different tsetse species require traps that have been specifically designed for them. Thus it is important to match the trap to the tsetse species. Biconical (Challier and Laveissiere, 1973) or pyramidal (Gouteux and Lancien, 1986) traps seem the best for riverine species such as *G. palpalis* or *G. fuscipes*. For savannah flies of east Africa, such as *G. morsitansmorsitans* subspp and *G. pallidipes*, NGU (Brightwell *et al.*, 1987) or Nzi (Mihok *et al*., 2002) are preferred whereas for southern Africa the use of the Epsilon trap is recommended (Hargrove and Langley, 1990). For the *fusca* species *G. brevipalis*, the H-trap is best whereas the NGU and Epsilon traps have been used successfully to catch *G. longipennis* in Kenya and Somalia (Kappmeier, 2000b). Trap performance efficiency is affected by several factors: vegetation type (Hargrove and Vale, 1980), location relative to vegetation (Dransfield et al., 1982), host abundance (van Etten, 1981),

time of day (Brady, 1972), changes in weather (Turner, 1987) and the fly's physiological state (Rogers and Randolph, 1978). Trap catches for savannah tsetse flies can be improved considerably by dispensing odours near traps (Dransfield et al., 1986).

H-trap (named after Hellsgate, South Africa, http://www.tsetse.org/FAQ/nzi.html)

Nzi trap (named after Swahili name for fly, http://www.tsetse.org/FAQ/nzi.html)

NGU trap (named after Nguruman, western Kenya, Masiga et al., 2014)

Mono-screen trap (Abila et al., 2007)

(A) Pyramidal trap, (B) Modified pyramidal trap, (C) Monoscreen trap, (D) Biconical trap (kindly provided by Prof. de Deken).

Figure 1.6. Common traps used in tsetse fly sampling for surveying, monitoring and surveillance.

In addition to traps, fly-rounds and electric screens together with visual and odour attractants are widely used to sample tsetse flies for monitoring, surveillance, abundance and distribution studies (FAO/IAEA, 2003). Tsetse fly collections can be made by cross-sectional or longitudinal sampling. Longitudinal sampling is done to study the population dynamics and behaviour of the tsetse fly with respect to the spatial (ecological zones) and seasonal variations of the

environment. Cross-sectional sampling of tsetse flies is done to study their distribution, determine their trypanosome infection rate or the effectiveness of control measures (Rogers and Randolph, 1978). Furthermore, electric screens are designed to evaluate the effectiveness of traps, target screens and odours (Vale, 1974a&b).

Currently, spatial and temporal tsetse fly count data are collected by fly-round, traps, and electric nets and are processed and analyzed using GIS along with remote sensing (RS). However, it is also possible to predict tsetse distribution without conducting entomological surveys using statistical models with climate and environmental data as predictor variables (Rogers and Williams, 1993). The use of GIS-RS helps: *(i)* to picture abundance, distribution and mortality rates of tsetse flies over space and time (Rogers and Randolph,1986; Hendrickx et al., 1999), *(ii)* to understand the seasonal tsetse fly population growth and movement (Rogers et al., 1996), *(iii)* to find favorable fly habitats in inaccessible sites using site specific spatial models (Rogers and Williams, 1993; Robinson et al. 1997; DeVisser et al., 2010; Matawa et al., 2013), *(iv)* to target control campaigns in areas with high tsetse fly densities (Hendrickx et al., 1999; Symeonakis et al., 2007), *(v)* to determine the number and location of fly suppression traps in a local control program (Symeonakis et al., 2007), *(vi)* to identify the direction and source of re-invasion during control interventions (Symeonakis et al., 2007).

Serological assays to detect antibodies in animals against tsetse fly bite (saliva) is another tool for monitoring and surveillance of tsetse flies. Studies using various tsetse fly species have shown that tsetse salivary proteins are immunogenic in mice, rabbits, cattle and humans with the induction of antibodies (Ellis et al., 1986; Caljon et al., 2006; Poinsignon et al., 2007 & 2008; Dama et al., 2013a & b; Somda et al., 2013; Caljon et al., 2014 & 2015). Whole saliva was extracted from *G. m. centralis* (Ellis et al., 1986), *G. m. morsitans* colony (Caljon et al., 2006 & 2014; Van Den Abbeele et al., 2007), *G. f. fuscipes*, *G. tachinoides*, *G. palpalis gambiensis* and *G. m. morsitans* colonies (Poinsignon et al., 2007), *G. f. fuscipes* colony (Poinsignon et al., 2008), the *G. p. gambiensis* colony (Dama et al., 2013a), from *G. p. gambiensis*, *G. m. submorsitans* and *G. tachinoides* colonies (Somda et al., 2013) and from *G. f. fuscipes*, *G. p. gambiensis*, *G. m. morsitans* and *G. pallidipes* colonies or imported pupae (Zhao et al., 2015) and was used as coating antigen of the assays with differing results. The use of whole saliva as coating antigen presents several limitations such as problems of mass production, reproducibility and specificity. To overcome this problem, the specific or recombinant protein based ELISA assay can be used in the immunoassay allowing large scale monitoring of host exposure to tsetse flies. Accordingly, a number of specific/recombinant candidate proteins of the immunogenic tsetse fly salivary protein

families are under investigation to develop a test that detects tsetse-host contact (host exposure to tsetse bite). The identified and evaluated potential synthetic salivary proteins of tsetse fly include recombinant tsetse salivary gland (rTsal) protein (Caljon et al., 2014 & 2015), tsetse salivary gland growth factor-1 (rTSGF-1) (Dama et al., 2013b), rTsal1, rTSGF -1 and rTSGF-2 (Zhao et al., 2015).

1.4. Distribution and abundance of trypanosomosis and tsetse flies

1.4.1. Distribution and abundance of trypanosomosis

The distribution of HAT is very much localized and occurs in specific focal areas in 20 countries. AAT, however, is widespread in 38 sub-Saharan African countries (Namangala and Odongo, 2014). The geographical distribution of trypanosome species varies across sub-Saharan Africa. *T. b. rhodesiense* occurs in East Africa and *T. b. gambiense* in West Africa. *T. congolense* tends to dominate in East Africa and *T. vivax* in West Africa although the two species are present in both regions (Namangala and Odongo, 2014). Accordingly, *T. congolense* accounts for more than 80% of AAT in domesticated animals in Southern, East and Central Africa and the losses associated with the disease (Van den Bossche et al., 2006; Namangala and Odongo, 2014). *T. congolense* Savannah type strains are the most pathogenic/virulent and spread all over the savannah ecosystem of sub-Saharan Africa (Bengaly et al., 2002; Namangala and Odongo, 2014) whereas *T. congolense* Forest occurs in humid forest ecosystems of West, Central and East Africa. *T. congolense* Kilifi is restricted to East Africa and to a lesser extent to Southern Africa (Mamabolo et al., 2009; Namangala and Odongo, 2014). In West Africa, *T. vivax* accounts for the majority of AAT cases (Namangala and Odongo, 2014). The distribution of trypanosomes in different hosts in sylvatic and domestic ecosystems is given in Table 1.1.

Environmental changes and encroachment of people influence the interactions between the host, parasite and vector (Van den Bossche et al., 2010). Encroachment of people causes environmental changes resulting in the development of three trypanosomosis niches: (i) sylvatic transmission cycle: tsetse-infested wildlife areas where livestock is absent or scarce. This is reservoir of tsetse flies and trypanosomes, (ii) endemic livestock trypanosomosis areas where the density of game animals is low and livestock constitute the main source of food for tsetse, and (iii) interface trypanosomosis, encroachment at the edges of the forest where people and livestock enter to sylvatic area routinely. Such interferences/interactions have repercussions for the distribution, density, dispersal and mortality rate (life span) of the vector, the species and strain

diversity, and epidemiology of the trypanosome, and the relative role of wild and domestic animals as hosts for tsetse and as reservoirs of trypanosomes (Van den Bossche et al., 2010). With gradual increase in encroachment, tsetse population significantly decreases and mechanically transmittable *T.vivax* tends to overtake. Currently, *T. vivax* is more and more becoming a severe problem in and outside the tsetse belt (Thumbi et al., 2010).

AAT is more common than HAT due to the fact that: (i) HAT is caused by either *T. b. rhodesiense* or *gambiense* but AAT is caused by more species, amongst them *T. brucei*, *T. vivax* and *T. congolense* (Makumi et al., 2000), (ii) HAT trypanosomes need more time to develop in the tsetse fly than the AAT trypanosomes, thus, the percentage of tsetse flies carrying HAT is much lower, (iii) *T. brucei*, *T. vivax* and *T. congolense* can infect animals in any part of Africa with a uniform distribution, but HAT is due to either *T.b. rhodiense* in East and Southern Africa or *T.b. gambiense* in Central and West Africa with a much more a focal distribution, (iv) the proportion of tsetse flies infected with any one of three AAT *Trypanosoma* spp. is greater than that with just one particular species of HAT (Woolhouse *et al*., 1993) and (v) finally, tsetse flies must feed on a host to infect it with trypanosomes. Several species of tsetse flies found in East and Southern Africa such as *Glossina pallidipes*, *G. morsitans*, *G. longipennis* do not prefer humans (Vale, 1974a&b; Makumi *et al*., 2000). This dispreference is generally more marked with older flies which are also more likely to be the infected. In contrast, livestock seems to be at least as attractive as wild hosts to tsetse flies. Indeed, the greater size and tolerance of cattle makes them better hosts than natural wild hosts such as warthogs. Consequently, tsetse flies are a more efficient vector of AAT than of HAT (Vale, 1977a&b).

1.4.2. Distribution and abundance of tsetse flies

Tsetse flies infest some 10 million km² of sub-Saharan Africa, extending from Mali and Ethiopia in the North, Senegal in the west to southern Somalia in the East and Angola and South Africa in the South. Tsetse flies are endemic in Africa between latitude 15° N and 29° S, from the southern edge of the Sahara desert to Zimbabwe, Angola and Mozambique (Ford, 1963; Murray et al., 1984; Reid et al., 2000; Wint and Rogers, 2000; Cecchi et al., 2008). Tsetse flies have three distinct habitats, namely, *(i)* savannah woodlands, *(ii)* the watercourses and drainage systems, and *(iii)* dense-forests (Vale et al., 2015). Almost all *morsitans* (savannah) group have exclusively invaded Eastern and Southern African deciduous savannah woodland surrounded by lowland forests and mostly transmits AAT whilst *G. longipalpis* is restricted to West Africa. *G. m. submorsitans* is exceptionally distributed from East to West Africa (Ethiopia to Senegal). The

palpalis group is distributed mainly in Central and West Africa and moderately in East Africa particularly invading evergreen woodland near water bodies, swampy areas, watercourses and drainage systems and transmit both AAT and HAT. With the exception of *G. brevipalpis* and *G. longipennis*, all *fusca* groups are associated with densely-forested regions of Central and West Africa. These differences in geographical distribution of tsetse species have important epidemiological implications (Ford, 1963; Wint and Rogers, 2000). Only "riverine" and "savannah" groups are epidemiologically important for HAT and AAT, respectively (Namangala and Odongo, 2014). The distribution of the 31 species and subspecies of tsetse flies in 38 African countries is compiled and summarized in Table 1.3 from Gouteux (1990), Jordan (1993) and Wint and Rogers (2000). In Tables 1.3, 1.4 and 1.5 it is shown that DR Congo is the most invaded nation in Africa with more than 18 tsetse species observed, followed by Cameroon (> 15 tsetse sp.) and Central Africa Republic (> 14 tsetse sp.). *G.m.submorsitans* is the most widely distributed tsetse species crossing the boundaries of at least 22 African countries.

1.5. Treatment and control of trypanosomosis and tsetse flies

1.5.1. Treatment and control of trypanosomosis

Trypanocidal drugs: There are currently only three trypanocides available for controlling AAT. They are on the market since 1960. The drugs are isometamidium and homidium, which have both prophylactic and therapeutic effects, and diminazene aceturate, which has only therapeutic properties (Leach and Roberts, 1981; Geerts et al., 2001). Unfortunately, there are 18 countries in which trypanocidal drug resistance has been reported (Delespaux et al., 2008) where 31.4%, 9.3% and 11.4% of *T. congolense* isolates from Africa are resistant to isometamidium, diminazene aceturate and both drugs (Geerts et al., 2001), respectively. The presence of drug resistance in the trypanosome population has been detected by a number of methods. Some of these include *(i) in vivo* monitoring sensitivity of mice inoculations for 60 days (Eisler et al., 2001), *(ii) in vivo* monitoring sensitivity of ruminant inoculations for 100 days (Eisler et al., 2001), *(iii)* molecular methods (Delespaux et al., 2008), *(iv)* sensitivity of in vitro cultured trypanosomes to trypanocides (Gray et al., 1993; Kaminsky and Brun, 1993). Measures should be taken to reduce the incidence of drug resistance and to minimize its impact and spread. According to the epidemiological context, five strategies are listed to minimize drug resistance development: *(i)* alternate the use of the trypanocides (sanative pair), *(ii)* limit treatment to clinical cases along with avoidance of drug misuse and under-dosing, *(iii)* monitor the quality of

drugs on the market, *(iv)* focus on tsetse control, and *(v)* rear of trypanotolerant livestock (Anene et al., 2001; Geerts et al., 2001).

Table 1.3. Global distribution of the 9 tsetse fly species and subspecies in the reverine tsetse group in 38 sub-Saharan African countries.

	G.p.gambeinsis Vanderplank, 1911	*G.tachnoides* Westwood, 1850	*G.p.palpalis* Robineau-Desvoidy1830	*G.pallicera. pallicera* Bigot, 1891	*G.caliginea* Austen, 1911	*G. pallicera newsteadi* Austen,1929	*G,fuscipes fuscipes* Newstead, 1911	*G,fuscipes quanzensis* Pires, 1948	*G,fuscipes martinii* Zumpt, 1935
West Africa									
Senegal	+								
Gambia	+								
Guinea-Bissau	+								
Guinea	+	+	+	+					
Sierra Leone	+		+	+					
Mali	+	+							
Liberia			+	+					
Cote d'Ivore	+	+	+	+					
Burkina Faso	+	+							
Ghana	+	+	+	+	+				
Togo	+	+	+						
Benin		+	+						
Niger		+							
Nigeria		+	+	+	+				
Chad		+					+		
Central Africa									
Cameroon		+	+	+	+	+	+	(+)	(+)
Central Africa		+	+	+	+	+	+		
Equatorial Guinea			+	(+)	(+)				
Gabon			+		+	+	+	(+)	(+)
Congo			+		(+)	+	+	+	(+)
DR Congo			+			+	+	+	+
East Africa									
Sudan		+					+		
Ethiopia		+					+		
Somalia									
Kenya					(+)		+		
Uganda							+		
Rwanda									+
Burundi									+
Tanzania							(+)		+
Southern Africa									
Angola			+			+		+	
Zambia							(+)		(+)
Malawi									
Mozambique									
Zimbabwe									
Botswana									
Namibia									
South Africa									
Swaziland									
No. nations infected	**10**	**15**	**15**	**8(1)**	**5(8)**	**6**	**11(2)**	**3(2)**	**4(4)**

(+) old reports, tsetse may not be present currently

Table 1.4. Global distribution of the 15 tsetse fly species and subspecies in the forest tsetse group in 38 sub-Saharan African countries.

	G.f.fusca walker, 1849	*G.n.negrofusca hopkinsi* VanEmden,1944	*G.tabaniformis* Westwood, 1850	*G.medicorum* Austen, 1911	*G.f.congolensis* Newstead & Evans, 1921	*G.haningtoni* Newstead & Evans, 1922	*G.fuscipleuris* Austen, 1911	*G.nashi* Potts, 1955	*G.schwertzi* Newstead & Evans, 1921	*G.frezili* Gouteaux, 1987	*G.severeni* Newstead, 1913	*G.venhoofi* Henrard, 1952	*G.n.negrofusca* Newstead, 1911	*G.longpennis* Corti, 1895	*G.brevipalpis* Newstead,1910
West Africa															
Senegal															
Gambia															
Guinea-Bissau	+														
Guinea	+	+	+							(+)			(+)		
Sierra Leone	+	+											(+)		
Mali															
Liberia	+	+		+									(+)		
Cote d'Ivore	+	+	+	+									(+)		
Burkina Faso				+											
Ghana	+	+	+	+	+								(+)		
Togo	+			(+)						(+)					
Benin				+	+										
Niger															
Nigeria	(+)	+	+	+	+	+							(+)		
Chad															
Central Africa															
Cameroon	(+)	+	+		+	+	+	+	+	(+)			+		
Central Africa		+	+	(+)	+	+	+	+	+						
Equatorial Guinea			+		+	+									
Gabon			+	(+)	+	+		+	+	+					
Congo			+		+	+		+	+	+					
DR Congo	(+)	(+)	+	(+)	+	+	+	(+)	+		+	+	+		+
East Africa															
Sudan	(+)				+		+							+	
Ethiopia														+	
Somalia														+	+
Kenya							(+)							+	+
Uganda		(+)	(+)	+	+		+							+	+
Rwanda															(+)
Burundi															(+)
Tanzania							(+)							+	+
Southern Africa															
Angola			+			+		+	+	(+)					
Zambia															+
Malawi															+
Mozambique															+
Zimbabwe															(+)
Botswana															
Namibia															
South Africa															(+)
Swaziland															
No. nations infected	**7(4)**	**8(2)**	**11(1)**	**7(4)**	**12**	**8**	**6(2)**	**5(1)**	**6**	**2(4)**	**1**	**1**	**2(6)**	**7**	**8(4)**

(+) old reports, tsetse may not be present currently

Table 1.5. Global distribution of the 7 tsetse fly species and subspecies in the savannah tsetse group in 38 sub-Saharan African countries and total tsetse species noted per nation.

								F+R+S
	G.m.submorsitans Newstead,1910	*G.longpalpis* Wiedemann,1830	*G.pallidipes* Austen, 1903	*G.m.centralis* Machado,1970	*G.austini* Newsead 1912	*G.swynnertoni* Austen, 1923	*G.m.morsitans* Westwood,1850	Total tsetse recorded in the country
West Africa								
Senegal	+	+						3
Gambia	+	(+)						2(3)
Guinea-Bissau	+	+						4
Guinea	+	+						9(11)
Sierra Leone	+	+						7(8)
Mali	+	(+)						3(4)
Liberia								5(6)
Cote d'Ivore	+	+						10(11)
Burkina Faso	+	(+)						4(5)
Ghana	+	+						12(13)
Togo	+	+						6(8)
Benin	+	+						6
Niger	+							2
Nigeria	+	+						11(13)
Chad	+							3
Central Africa								
Cameroon	+	+						15(19)
Central Africa	+	(+)						14(16)
Equatorial Guinea								4(6)
Gabon								10(13)
Congo								10(12)
DR Congo	+	+	+	+				18(23)
East Africa								
Sudan	+		+					7(8)
Ethiopia	+		+					5
Somalia			+		+			4
Kenya	+		+		+	+		7(9)
Uganda	+		+	+				9(11)
Rwanda			+	+				3(4)
Burundi				+				2(3)
Tanzania			+	+	+	+	+	
Southern Africa								
Angola				+				8(9)
Zambia			+	+			+	4(6)
Malawi			+				+	3
Mozambique			+		+		+	4
Zimbabwe			+		(+)		+	2(4)
Botswana				+				1
Namibia				+				1
South Africa					+			1(2)
Swaziland					+			1
No. nations infected	**22**	**11(4)**	**13**	**9**	**6(1)**	**2**	**5**	

(+) old reports, tsetse may not be present currently; F = Forest tsetse group, R = Riverine tsetse group, S = Savannah tsetse group

Herbal medicine: in some regions, medicinal plants are also used by livestock owners for trypanosomosis treatment. In this regard, a review indicated that 264 plant species belonging to 79 families are investigated in Africa. Of these, 48 bioactive anti-trypanosomal compounds are successfully isolated in pure form, and 18 have been shown to be effective against trypanosome-induced pathological changes (Ibrahim et al., 2014). Although some anti-trypanosomal activity is described, none of those treatments seems to be in a position to broaden its use in the future.

Nanobody: experimental nanobody-based interventions have been shown to kill trypanosomes through conjugating the human serum lytic factor (i.e. apolipoprotein L1 molecule) to nanobodies (Baral et al., 2006), and through the production of specific nanobodies against trypanosome's VSG. The anti-VSG specific nanobodies cause very rapid immobilisation of the parasites, massive enlargement of the flagellar pocket, major blockade of endocytosis, reduced intracellular ATP-levels, loss of mitochondrial membrane potential and finally death of the trypanosome in mice (Stijlemans et al., 2011). Albeit promising, this strategy remains confined to laboratory experiments so far, thus it is unlikely to be available soon for field use.

Immunization and immunobiology: At present, vaccine development was not successful due to antigenic variation of the trypanosomes and trypanosome-induced adaptive immunity dysfunction of the host. Cattle immunization using cysteine peptidases (congopain) induced a significant improvement of anemia and immunosuppression during infection, but its protective efficacy is low. Injection of glycosyl-inositol-phosphate anchor protein before trypanosome infection was successfully alleviating clinical symptoms such as weight loss, liver damage, acidosis and anemia (Antoine-Moussiaux et al., 2009; La Greca and Magez, 2011). More than 10 vaccine candidates were reported in the literature such as a flagellar pocket antigen, invariant surface glycoprotein, glycoproteinphosphatidylinositol, cystein protease, actin, tubulin, sialidase, cation ATPases and others resulting in partial/no protection (Antoine-Moussiaux et al., 2009; La Greca and Magez, 2011). The development of an effective, affordable vaccine for wide use in the field in the near future is unfortunately very unlikely.

Breeding trypanotolerant livestock breeds: The ability of an infected animal to maintain PCV (Murray et al., 1990), grow (Van der Waaij et al., 2003) and reproduce (Trail et al., 1993), following either experimental or field infection, could be used as a method for identifying trypanotolerant individuals. N'Dama and West African Shorthorn breeds of West and Central Africa (Murray et al., 1990), two shorthorn taurine Lagune and Baoulé breeds (Berthier et al., 2015), Sheko and Horro cattle breeds of Ethiopia (Lemecha et al., 2006) possess a significant

degree of tolerance to trypanosomosis. They resist the development of anemia in the face of infection (Murray et al., 1990). This could permit rational breeding programmes to be instituted.

1.5.2. Tsetse fly control methods

1.5.2.1. Game animal killing and bush clearing

Wildlife and man have lived side-by-side for thousands of years (Lamprey and Reid, 2004). Wildlife serves as a reservoir for trypanosomes and blood meal for tsetse flies. Thus, game destruction and bush clearing have been used in the past to eradicate tsetse fly populations (Matthiessen and Douthwaite, 1985). Between the 1920s and 1960s, attempts to eradicate the tsetse flies resulted in the slaughter of 1.3 million game animals and extensive bush clearing, which permanently destroyed the wildlife habitat (Matthiessen and Douthwaite, 1985). Game destruction as a method of tsetse fly control is now considered unacceptable from an ecological point of view (Hargrove, 2003b). Game destruction was replaced by complete and partial (selective or discriminative) bush clearing. Again, the latter is replaced by blanket aircraft-assisted or ground insecticides spraying, which was further refined by discriminative application of insecticides assisted by tsetse fly density surveys. The latter one seems more precise, economical and effective (Hocking et al., 1963). However, the overall tsetse fly control and eradication effort is a hot and controversial topic of debate among ecologists. Their fear emanates from changes in human population and land jeopardizing biodiversity (Lamprey and Reid, 2004). Such agricultural development and settlement leads to tsetse eradication by itself (Habtemariam et al., 1983). This implies that game destruction and bush clearing is still going on, but in an indirect way (Hargrove, 2003b).

1.5.2.2. Aerial spray

There are a number of compounds toxic for the tsetse fly. In the group of organochlorides, dieldrin, with a median lethal dose of 10 ng per fly, is about twice as toxic as gamma-HCH, and more than 8 times as toxic as DDT to *Glossina austeni*. In the group of synthetic pyrethroids, resmethrin is effective at a median lethal dose of 4 ng to *Glossina austeni*. A number of organophosphate compounds are also toxic to tsetse fly. The most toxic compound of this group is fenthion at a median lethal dose of 8 ng per fly. The other effective organophosphates are chlorfenvinphos (12 ng per fly), tetrachlorvinphos (20 ng per fly), and dichlorvos (20 ng per fly). Malathion (95% purity) is nontoxic at the relatively high dosage of 150 ng per fly. In the group of carbamate compounds, propoxur (12 ng per fly) is the most effective insecticide for tsetse fly

(Hadaway, 1972). Between 1960 and early 1970s DDT, dieldrin, endosulfan, decamethrin, 2 methyle-4, 6-dinitro-ortho-oresol, delthametrin alone or mixed have been used as ground spraying and between 1970-1990 as sequential aerosol technique (SAT) at different times in different African countries. Gradually, very low dosages of endosulfan (non-residual), and later a cocktail of endosulfan and synthetic pyrethroid insecticide were used to minimize the residual side effects of persistent insecticides on untargeted pest and environment. Between 1990-2000 traps and targets were widely used in Africa using synthetic pyrethroid insecticides, particularly deltamethrin suspension concentrate formulation of 0.6% (w/v) (Kurugundla et al., 2012).

From 2000 onwards sequential aerial spraying (SAT) of deltamethrin 0.26–0.3 g/ha in Botswana (Kgori et al., 2006; Kgori et al., 2009; Kurugundla et al., 2012) and 0.33–0.35 g /ha in Ghana (Adam et al., 2013) have been reintroduced since the adopted target approaches between 1990-2000 are proving difficult to effectively implement due to accessibility problems and fly reinvasion (Kurugundla et al., 2012). The insecticide is applied at night using four turbo thrush fixed-wing aircraft. The aircrafts are all guided by previously unavailable advanced GPS-GIS navigation guidance system and spray management equipment accurate to about 1 m which could ensure precision placement of spraying material and eliminate overdosing or even under-dosing through erratic track guidance (Kgori et al., 2006; Kgori et al., 2009; Kurugundla et al., 2012; Adam et al., 2013). Use of modern GPS-guided navigation and spray systems makes this tool very effective for area-wide tsetse suppression in dense humid forest ecosystems (Van der Vloedt et al., 1980) or eradication in open savannah-type ecosystems (Kgori et al., 2006). Aircrafts or helicopters are used to spray a mist of ultra-low volume of non-residual insecticides 10–15 m above the tree canopy in 5–6 subsequent spraying cycles separated by 16–18 days depending on the temperature (Allsopp and Hursey, 2004). Besides environmental concerns, the main limitation of large-scale aerial or ground spraying is that there is little or no protection from re-invasion once the initial control operation is completed and the gains achieved in reducing tsetse fly densities can be rapidly lost (Hargrove, 2003b). If a controlled area is subject to re-invasion from all sides, then a treated block of 100 km² is completely re-infested within a year. Therefore, targets, traps, insecticide-treated cattle or some combination of the three have to be deployed in cleared areas to prevent re-invasion (Hargrove, 2000). In Botswana, the crossing of tsetse flies (reinvasion) from one unsprayed area to another sprayed area between the successive SAT spray operations has been avoided by integration of insecticide impregnated targets (Kgori et al., 2006; Kgori et al., 2009; Kurugundla et al., 2012). In Ghana, SAT has resulted in 98% reduction in tsetse but failed to eliminate beyond this as fly density is too low. To overcome this, an integrated

strategy of intervention has been adopted, whereby the SAT has been complemented by ground spraying, insecticide treated targets (ITT) and insecticide treated cattle (ITC) (Adam et al., 2013).

Despite the insecticides being in use since a long time, resistance to the insecticides in tsetse flies has not been reported yet. One study compared the resistance and susceptibility within and between tsetse species for topical application of deltamethrin. Three solvents for deltamethrin (0.08 ng in 0.5 µl) topical application were compared to kill tsetse flies. These solvents are acetone, butanone, 2-ethoxy-ethanol and di-isobutylketone. Different tsetse fly species has displayed different level of innate resistance and susceptibility to deltamethrin dissolved using in different solvents as measured by lethal dose of 50% of the population (LD_{50}) 48 hours after application. Accordingly, tsetse resistance is ranked in the order of *G. p. gambiensis* > *G. p. palpalis* > *G. austeni* > *G. m. morsitans* using acetone solvents; *G. p. pallidipes* > *G. tachnoides* > *G. m. morsitans* using butanone, 2-ethoxy-ethanol solvents; and *G. p. palpalis* > *G. austeni* > *G. m. morsitans* using di-isobutylketone. Apart from tsetse fly species, the resistance of tsetse flies have been ranked in the order of fed flies > starved flies; pregnant > non-pregnant female flies and mature males > young male flies (de Deken et al., 1998).

1.5.2.3. Stationary baits (traps and targets)

Traps are important for controlling and monitoring tsetse flies (Green, 1994). Odour-baited insecticide impregnated traps or targets have been used to eliminate tsetse flies or as a barrier to re-invasion (Hargrove, 2003b). The advantages of this method are that it can be easily extended, i.e., using more traps, according to tsetse fly density, dispersal and seasonal dynamics, that it is cheap and unsophisticated and suitable for local deployment by farmer communities to protect small areas (Hargrove, 2003b). Interest in bait technology was stimulated by the discovery of the low reproductive rate of tsetse flies, so that as few as four such targets per square kilometre is sufficient to eliminate isolated populations of *G. pallidipes* Austen and two sub-species of *G. morsitans* (Hargrove and Vale, 1979; Hargrove, 1988; Hargrove, 2003a & b).

Pyrethroids used for impregnation of the baits include deltamethrin, alpacypermethrin and beta-cyfluthrin (Torr, 1985). The dose varies according to the insecticide and the period of persistence. However, a 1% suspension of deltamethrin applied to cotton cloth (Torr *et al*., 1992), 1% beta-cyfluthrin and 2% alphacypermethrin (Mangwiro et al., 1999) will be effective for at least a year, irrespective of rainfall or temperature. Treating a target with suspension concentrate formulations of 0.4% deltamethrin, or 0.4% beta-cyfluthrin or 0.8% alphacypermethrin produces high (>70%)

mortalities for nine months and 0.8% formulations of beta-cyfluthrin and deltamethrin are effective for more than a year (Mangwiro *et al.*, 1999).

Recently, a miniature (0.06m²) blue–black target has been deployed and appears to be more cost-effective (Lindh et al., 2009). Deployment of these tiny targets at a full scale (overall target density 5.7/km²) over 500 km² in Northern Uganda suppressed the tsetse fly population by more than 90% in 12 months. As a guide, a mathematical model suggested that a 72% reduction in tsetse population is required to stop transmission in those settings (Tirados et al., 2015). The tiny target is effective against four species of tsetse fly (*G. tachinoides* and *G. palpalis gambiensis* (Burkina Faso), *G. fuscipes quanzensis* (Democratic Republic of Congo), *G. f. martinii* (Tanzania) and *G. f. fuscipes* (Kenya). This has important implications in making the costs of control of the riverine group of tsetse vectors of HAT and AAT an affordable option (Esterhuizen et al., 2011).

The trap and target method is reported to have a number of limitations for its efficient application, namely *(i)* the importance of trap/target deployment and siting demands expertise, *(ii)* the maintenance requirements of traps and targets, *(iii)* their periodic replacement, *(iv)* the periodic replenishment of the odours, *(v)* the use of cloth material with an appropriate reflectivity pattern, *(vi)* insecticide deposits degrading by UV light and *(vi)* the use of the most suitable trap/target in relation to tsetse fly species and geographic distribution, *(vii)* in certain dense habitats the use of these devices over large areas is uneconomic (Vreysen, 2001).

1.5.2.4. Insecticide-treated cattle (pour-ons)

The insecticides applied to cattle to control tsetse do not repel tsetse flies and thus do not protect individual animals (Baylis *et al.*, 1994; Vale *et al*., 1999). However, each flies that bite - or even touches - a treated animal will die within a few hours. Tsetse flies seem to feed preferentially on the few individuals in a herd particularly older and larger cattle in the herd (Torr et al., 2001). As a general rule, only half the herd needs to be treated, especially the bigger animals should be treated with insecticides (Torr et al., 2007a), particularly on their legs since tsetse flies land most often on the legs (Bouyer et al., 2006; Torr et al., 2007b). Such restricted use is cheap and safe as it saves insecticide by 80% compared to its application to the whole body of the animal (Bourn et al., 2005).

Insecticide-treated cattle should be herded in areas with high tsetse fly concentration at times of the day when the flies are active to ensure maximum contact with the tsetse population. In most

seasons tsetse are active in the first few hours after sunrise and the last hour or so before sunset. In the cool season, the peak activity occurs in the middle of the day. For *G. fuscipes* and *G. palpalis*, the middle of the day is optimal in all seasons. The advantages of this method are that *(i)* it does not require maintenance, *(iii)* it is amenable to integrated control of tsetse, *Stomoxys* and ticks, *(iv)* it reduces the amount of insecticides used and treatment time by 80-90%, with a subsequent reduction of their environmental impact through partial application to legs and older animals (Bourn et al., 2005). Attention should be drawn here on the risk of interrupting the endemic stability of tick borne diseases when controlling tsetse flies intensively. Drastic clearing of ticks from animals renders the animals immunologically incompetent to tick-borne diseases (TBD) which subsequently results in severe TBD outbreaks and high calf mortality due to endemic instability.

The disadvantages of the pour-on method are that *(i)* sufficient cattle density is required, *(ii)* the large proportion of the herd that requires treatment, *(iii)* applicable only for tsetse fly species whose host preference is livestock, *(iv)* high treatment frequency, *(v)* the high cost of the insecticides, *(vi)* insecticide residues in cattle dung negatively affects dung fauna (Vale et al., 2002), *(vii)* sustainability in the motivation and participation of farmers and *(viii)* the potential development of resistance to the insecticides in both tsetse and ticks (Vreysen et al., 2013), *(ix)* increase in AAT at the start due to intentional grazing of treated cattle in heavily infested areas (Baylis *et al*., 1994; Vale *et al*., 1999).

In addition, repellent odours can also be used as they have the potency to protect humans and animals against tsetse fly bites when applied onto the host. These include 2-methoxyphenol, cetophenone, lactic acid, and naphthalene (Voskamp et al., 1999). The use of repellents is intellectually attractive but still did not prove to be a method of choice in real life conditions.

1.5.2.5. Sterile insect technique (SIT)

SIT is an important tsetse eradication tool (Kabayo, 2002). The application of SIT for tsetse control has been suggested long time ago (Knipling, 1955). Tsetse flies appear highly susceptible to SIT because of their low reproduction rate. Successful application of SIT could, in principle, eradicate tsetse flies without the environmental costs of habitat destruction and insecticidal use (Knipling, 1955). The advantages of SIT are that it is *(i)* not intrusive to the environment, *(ii)* has no adverse effects on non-target organisms, *(iii)* is species-specific, *(iv)* can easily be integrated with biological control methods such as parasitoids, predators and pathogens, *(v)* no evidence of development of resistance to the effects of the sterile males (Vreysen et al., 2013). SIT as a

technique is promising, but needs preconditions (both in the factory and in the field). The preconditions include (i) prior tsetse population suppression in the field by targets, SAT or other, (ii) efficient mass-rearing and irradiation for male release, (iii) survival, host seeking, feeding and adaptation of the released male under field condition, (iv) ensure reproductive performance and competitiveness to mate wild female tsetse flies (Lees et al., 2015). Ethiopia started the SIT programme in 1996 but released only recently irradiated male tsetse flies.

1.5.2.6. Some other tsetse control options

Many species of tsetse flies are infected with a virus that causes salivary gland hypertrophy (SGH), and flies with SGH symptoms have a reduced fecundity and fertility. The prevalence of SGH in wild tsetse fly populations is usually very low (0.2%–5%), but higher prevalence rates (15.2%) have been observed occasionally. A *Glossina pallidipes* colony originating from Ethiopia was successfully established in 1996, but later up to 85% of adult flies displayed symptoms of SGH. As a result, the tsetse colony at Kaliti was devastated by 2002 (Abd-Alla et al., 2011). All males with enlarged glands are sterile. The evidence obtained shows that the tsetse virus may be used in biological control of *G. pallidipes* (Odindo, 1988). In wild tsetse populations the virus is essentially transmitted vertically from mother to progeny. Horizontal transmission of the virus is not recorded through contact between flies, mating, faecal contamination or during feeding on animal hosts. Another option is infecting tsetse flies with a sexually transmissible bacterial disease of tsetse, called *Wolbachia*, which causes an embryonic mortality of tsetse larva. This bacteria can be cultivated in the laboratory and integrated to SIT. The irradiation males ready for release can be infected intentionally to ultimately infect the wild female flies venerally (Lees et al., 2015). Furthermore, more than 90% of a purified virus suspension loses its infectivity after 3 days at 4°C. The mass production of the virus for field application and its formulation is not feasible practically. Artificial infection of healthy flies by feeding on contaminated blood is efficient, but the symptoms of SGH corresponding to highest virus loads are observed only in the next generation. The above technical challenges and difficulties make the use of this virus as a biological control agent to control tsetse flies impractical unless an efficient way to deliver the virus to the target tsetse flies is in place (Abd-Alla et al., 2011).

Avoidance of grazing areas where tsetse are abundant reduces tsetse fly bites. Concentrating cattle in highland areas or in habitats where they are less abundant are widely utilized. Use of wood smoke reduces the numbers of flies around their cattle. Physical barriers such as netting

prevent tsetse flies from entering the cattle grounds. This method is only effective for owners of zero-grazed cattle (Ford, 1971). Rancid butter and camel fat are used as a tsetse repellents in Somalia but to date there is no good evidence that these chemicals can be used to reduce the numbers of tsetse flies biting cattle (Torr *et al*., 1996).

1.6. Tsetse and trypanosomosis situation in Ethiopia

In Ethiopia, five tsetse fly species, namely, *G. pallidipes*, *G.m.submorsitans*, *G.f. fuscipes*, *G. tachnoides* and *G. longipennis* have been reported (Langridge, 1976; Jordan, 1993) to be involved in the transmission of *T. congolense*, *T. vivax* and *T. brucei* (Langridge, 1976). Of 1.1 million km² land area, about 240,000 km² of fertile area are under threat of tsetse-transmitted-trypanosomosis. Therefore, vegetation clearing, insecticides, trypanocidal drugs, and settlement (or resettlement) have been recommended as the most effective and feasible tsetse control method (Habtemariam et al., 1983). Subsequently, several interventions were undertaken in the field. The interventions included massive deforestation/resettlement and domestic animals development programs in Gambella after 1985 (Nigatu et al. , 1992; Hadis et al., 1995), mono-screen trap deployment at Didesse valley (Slingenbergh, 1992), a pour-on study at Gibe valley (Leak et al., 1995), insecticide-impregnated targets at Gibe valley (Leak et al., 1996), biconical and NGU trap deployment at upper Didessa valley (Belete et al., 2004) and Nzi, epsilon, pyramidal, NGU, biconical and canopy traps study at Chanka in west Wollega in 1997 (Mihok et al., 2007). Insecticide (deltamethrin) impregnated targets and odor baited traps with acetone, octenol and cow urine combination have been used widely in controlling tsetse flies in Ethiopia (Slingenbergh, 1992; Belete et al., 2004). Furthermore, a tsetse mass rearing factory has been launched in 1996 for the release of sterile males to eliminate tsetse from the southern rift valley (Alemu et al., 2007). Over the last three decades, dynamic changes are noticed in tsetse infested areas of Ethiopia particularly with changes in ecology, climate, land use, settlement of huge stocks of animals and human population (Reid et al., 2000). In the face of impersistent control efforts, trypanosomes have devised a coping strategy such as drug resistance and mechanical transmission by biting insects whilst tsetse frequently re-invades tsetse fly suppressed areas. In this regard, a widespread occurrence of multi-drug resistant trypanosomes has been reported at Gibe valley (Leak et al., 1996; Mulugeta et al., 1997; Rowlands et al., 2001; Moti et al., 2012), at north western Ethiopia (Afewerk et al., 2000; Dagnachew et al., 2015b), at north Omo valley (Assefa and Abebe, 2001) and at upper Didessa valley (Tewelde et al., 2004). In Gibe valley,

prescription of trypanocides led to a significant economic return from treating animals despite the high prevalence of drug resistance of the trypanosomes (Itty et al., 1995).

In Ethiopia, tsetse-transmitted animal trypanosomosis (AAT) has never been reported so far in central, northern, north-eastern and south-eastern Ethiopia. The remaining part of the country is too high (central and north Ethiopia) or too arid (east and south Ethiopia), thus not suitable for tsetse flies. Tsetse flies prevail only in the west, south-western Ethiopia and to some extent in the southern rift-valley. Tsetse fly and trypanosomosis issues has been dealt with nationally by the national tsetse and trypanosomosis investigation and control center (NTTICC) at Bedelle. In 1996 a southern tsetse eradication project (STEP) was launched that was mandated to eradicate tsetse flies from the southern rift valley using the sterile male technique. In 2014, NTTICC and STEP were merged to become the national institute for the control and eradication of tsetse and trypanosomosis (NICETT). In general, the available studies on the prevalence of trypanosomosis and tsetse density in Ethiopia are fragmented and concentrated in three valleys: Didessa valley, Gibe valley, southern rift valley and north western Ethiopia. On the other hand, other prominent valleys such as Anger, Asosa , Baro-Akobo, Birbir, Birsheleko and the rest valleys in south western Ethiopia are not adequately studied.

1.6.1. Didessa valley

Using targets and traps in a region comprising 850 km^2 of the upper Didessa valley, it was cleared of tsetse flies during 3 years (1986-1989) (Jemal and Hugh-Jones, 1995; Slingenbergh, 1992). In this regard, a rapid decline of *G. m. submorsitan* population (>95%) was noticed from 25 flies/trap/day to below the detectable level within four months after the operation had begun in 1986. The density of tsetse flies was reduced from 32 flies/trap/day to very low in an operation exclusively aimed at *G.tachinoides* along Didessa river banks (Slingenbergh, 1992**)**. The prevalence of trypanosomosis in cattle dropped dramatically from 60% to nearly 1% in all areas of Didessa valley (Jemal and Hugh-Jones, 1995).

1.6.2. Gibe valley

In Gibe valley, vector control (pour-on and insecticide impregnated targets) and parasite control by mass treatment with diminazene aceturate before tsetse control were implemented from March 1986 to March 1998. Both the vector and parasite control approaches were associated with significant decreases in both incidence and persistence of trypanosome infection. However, despite the regular treatment with diminazene aceturate, the duration of infection increased

during the study. This could be due to the occurrence of new infections or the persistence of existing infections (Schukken et al., 2004). In Gibe valley, from 1991 to 1993 between 2,000 and 4,000 cattle were treated at monthly intervals with a synthetic pyrethroid insecticide (i.e. cypermethrin) at a dosage of 1 ml per 10 kg bodyweight to control tsetse flies. Treatments were given as pour-on application along the backlines of animals, using automatic drench- gun applicators. This resulted in a decline in the apparent density of *G. pallidipes* by 93% and of *G. m. submorsitans* by 83%. This reduction was associated with a reduction in trypanosome prevalence in cattle of over 74% in 1993, despite a high level of resistance to all available trypanocidal drugs. The numbers of *Stomoxys* spp. and Tabanidae were also significantly reduced (Leak et al., 1995). In the same valley *G.pallidipes* reduced by 80.5 % from 2.1 to 0.41 flies/trap/day in the 12 months of tsetse fly control using deltamethrin impregnated targets (Leak et al., 1996). Results of four years on systematic trypanocidal chemotherapy superimposed by tsetse control provided reductions of 95% and 75% in the mean relative densities of tsetse and biting flies, respectively, and of 63% in the prevalence of trypanosomal infections in cattle. Despite these reductions, there was no significant increase in the body weight of the cows, calving rate or the mean body weight of calves at 12 months of age. There was, however, an average decrease of 57% in calf mortality (including still births) by 12 months of age, an increase of 49% in the ratio of live calves under 12 months of age to cows over 36 months of age, and an increase of 8% in the body weight of adult males (Rowlands et al., 1999). In 1990 a tsetse and trypanosomosis control programme was spoiled by the theft of a large number of the targets due to a lack of local involvement. Local involvement is needed to stop theft, to guard the targets and detecting thieves and to contribute money or labour. A local participatory research approach can generate practical results in the provision of local public goods (Swallow and Woudyalew, 1994). Despite control efforts, there was a report that tsetse flies advanced to previously uninvaded new locations (Leak et al., 1993).

Similarly, the suppression of tsetse using odor baited mass trapping technology was conducted over 10 years (1995-2005) in Luke area of Gibe valley. The benefits of the suppression efforts include (*i*) adult fly catches decreased to negligible number, (*ii*) trypanosomosis reduced from 29% to 10%, (*iii*) cattle populations increased 5 fold, (*iv*) milk production increased 13 fold from (0.1 to 1.3 L per day), (*v*) number of oxen used as draft animals increased from 3 to 136 (45.3 fold), (*vi*) area of cultivated land increased from 30% of the total area from 12 ha to 500 ha (53% increase) and (*vii*) household income increased from 15.6 USD in 1995 to 60 USD in 2005 (Baumgärtner et al., 2008). Since 1996, tsetse control using odor-baited traps have been carried

out in the Luke area of Gurage zone, southwestern Ethiopia. *G. m. submorsitans* was the dominant fly, but *G.f. fuscipes* and *G. pallidipes* were also recorded. A more efficient control system was developed between 2002 to 2004 by *(i)* improving the skill of the communities to conduct entomological monitoring themselves and *(ii)* in parallel by introducing experts of geostatistical methods to identify, monitor, kriging and mapping the spatiotemporal dynamics of patches with increased fly densities, referred to as hot spots. To respond to hot spots, the community was advised and assisted in control trap deployment. This community-expert assisted control method was more efficient as the number of traps was reduced to 127 (107 monitoring traps and 20 control traps) compared to the previously 216 traps used to mass trapping system while maintaining the previously reached levels of tsetse occurrence and trypanosomosis prevalence (Sciarretta et al., 2005).

1.6.3. North western Ethiopia

In the Awi zone of north western Ethiopia 3,200 zebu cattle were examined for trypanosomosis. Of these, 322 (10.1%) were infected with trypanosomes. *T. vivax* was most prevalent (54.1%). The prevalence of trypanosome infection was not significantly different between age categories and sexes. The prevalence differed significantly between wet (higher) and dry (lower) season. The average PCV of cattle infected with trypanosomes (21.1%) was significantly lower than negative animals (26.0%) (Kebede and Animut, 2009). In the same region at Metekel zone, average tsetse fly (*G.tachinoides*) density decreased from 1.13 to 0.18 flies/trap/day after control using 0.1% deltamethrin sprayed targets deployed along the riverside via community participation. Subsequently, the prevalence of trypanosomosis was reduced from 12.14 % before to 3.61 % after control coincides with the tsetse fly reduction (Girmay et al., 2016). In several areas the reduction in tsetse fly is relatively higher than the reduction in prevalence of trypanosomosis suggesting that biting flies like tabanids and *Stomoxys* species could take an important role in the transmission of trypanosomes. Biting flies (tabanids and *Stomoxys)* have been incriminated for trypanosomosis transmission in non-tsetse infected area particularly for *T. vivax* (Cherenet et al., 2006; Sinshaw et al., 2006; Fikru et al., 2012). *Trpanosoma vivax* isolates from both tsetse infested and non-tsetse areas of north western Ethiopia showed a variety of virulence factors leading to the development of acute clinical signs, gross and histopathological lesions under controlled experimental infection of calves. However, the parasitemia and clinical signs appeared earlier (6-7 days post infection) in non-tsetse infected areas compared to tsetse-infested areas (12-14 days post infection) (Dagnachew et al., 2015a & b).

1.6.4. Southern rift valley

The southern tsetse eradication project (STEP) was launched in 1997 to eradicate tsetse fly from a 25,000km² area in the Southern rift valley. The STEP approach used insecticide-treated cattle (pour-on), insecticide impregnated targets, ground spray and finally once 95% suppression reached the sterile insect technique. The tsetse mass rearing facility in kaliti planned to increase the fly population from 100,000 to 700,000 - 1,000,000 *G.fuscipes* females as well as *G.pallidipes* (Desert, 2007). In the southern rift valley 460 deltamethrin (0.4%) impregnated targets were deployed at a density of 4 targets per km^2 in 2003-2004 which resulted in an 88.9% overall reduction of *G. pallidipes* from 1.35 flies to 0.05 flies per trap per day (Bekele et al., 2010). The incidence of trypanosomosis in sentinel cattle declined from 10% to 0.95% and the PCV improved from a mean of 24.1% to 27.2%. Alemu et al. (2005) reported a 92% reduction in the same valley. The effort resulted in an 83.25% reduction from 10.75% to 1.8% in the incidence of trypanosomosis in sentinel cattle. The corresponding packed cell volume (PCV) improved from a mean of 21.8% to 25.5%. Cattle owners in tsetse controlled area harvested daily milk yield of 26-27% more at the beginning, 25-29% more at the middle and 17-21% more at the end of the lactation period. In addition, cows in tsetse controlled area had the lactation length increased by 1.20 to 1.35 months; age at first calving was shortened by 5.30 to 5.10 months and calving interval was shortened by 4.20 to 3.20 months (Taye et al; 2012). Tsetse-challenge and associated trypanosomosis seriously lowered the number of female calves, pregnant cows and lactating cows (herd composition), increased mortality, reduced milk yield and reproductive performance of cattle (Tigicho et al., 2012). In another study, no trypanosome positive calves and no report of cattle mortality was reported in tsetse-controlled regions whilst 2.95 % trypanosome prevalence and 16.48 % calf mortality was reported in tsetse-uncontrolled area in 1 year time. The apparent densities of flies/trap/day in tsetse-uncontrolled area were 30-fold higher than in tsetse-controlled area . The mean bodyweight gain of calves in tsetse-controlled area (40.23 kg) was significantly higher than that of the uncontrolled area (34.74 kg). The relative number of calves in a herd tend to be higher in the tsetse-controlled area. The intervention by the STEP project has significantly reduced tsetse population and trypanosomosis consequently contributing to improved calf growth and survival (Gechere et al., 2012). In the southern Rift Valley of Ethiopia around Arba Minch the prevalence of cattle trypanosomosis was 7.2% (133/1838) with 66.9 and 33.1% of them accounted for by *T. congolense* and *T. vivax*, respectively. The number of flies caught per trap per day was 1.4 for *Glossina* species (*G. pallidipes* and *G.f fuscipes*) and 2.8 for other biting flies (Sheferaw et al., 2016).

1.6.5. Birbir valley

The spatio-temporal distribution of *G. m. submorsitans* and *G. pallidipes* at Ethiopia's Keto pilot site was used for precision targeting interventions in an adaptive pest management system. The method delineates hot spots on maps, measures shapes and sizes of patches, and discards areas with low tsetse density. Both tsetse species displayed an aggregated distribution characterized by two main patches in the south and an extended gap in the north. Spatial patterns were positively correlated and stable in most cases with the exception of the early dry season and the short rainy season when there were differences between the species and sexes. The method allows a better delineation of the territory for control operations and a more precise computation of the required number of the relatively expensive traps used for monitoring and control purposes (Sciarretta et al., 2010). A total of 384 flies of four species (*G.pallidipes, G.m.submorsitans, G.f.fuscipes* and *G.tachinoides)* were dissected in Birbir valley. Higher infection rates were observed in the *morsitans* group (5.46%) than in the *palpalis* group (0.52%). Higher proportion of tsetse flies was captured in the riverine vegetation type followed by *Savannah*, forest, bush, and cultivated areas (Desta et al., 2013). Three tsetse species (*G. pallidipes*, *G. m. submorsitans* and *G.f. fuscipes*), *Stomoxys* and tabanus were caught at Hawa Galan of Birbir valley. The overall apparent density of tsetse species caught was 10.5 flies per trap per day, with a higher proportion of female flies (57.2%). Of 389 cattle examined, 42 (10.8%) were infected with trypanosomes with 54.8% accounted for *T. congolense*, 23.8% for T. *brucei* and 21.4% for *T. vivax*. Poor body condition was a risk factor and 95.2% of cattle that were positive for trypanosomes had a PCV < 24% (Lelisa et al., 2014). *G. tachnoides*, *G. m. submorsitons* and *G. pallidipes* were the tsetse flies species caught along with other biting flies. Overall the prevalence of cattle trypanosomosis was 16.9% based on 585 blood samples examined using the buffy coat method. *T. congolense* was the dominant species (69.7%). The prevalence significantly varied among localities (PAs), body condition scores and PCV values (Getachew et al., 2014). Habte et al. (2015) reported a prevalence of 7.1% based on 650 blood samples also using the buffy coat method. *T. congolense* (82.61%) was the dominant trypanosome species followed by mixed infection of *T. congolense* and *T. vivax* (8.67%). Infection with *T. vivax* and *T. brucei* were equally prevalent 4.35% (2/46). Of 1132 cattle examined in Birbir valley in three districts, 97 (8.7%) were trypanosome positive with 82 cases (7.2%) accounted for *T.congolense*. The overall anemia (PCV < 24%) prevalence was 39.1% (443/1132) in which only 51 cases of the 97 trypanosome infected cattle had anemia (Tasew and Duguma, 2012) suggesting occurrence of other causes of anemia in the area.

A standardized questionnaire survey was employed on the farmers' perception on cattle trypanosomosis and tsetse fly in selected districts in Baro-Akobo and Gojeb river basins, South Western Ethiopia. A total of 94.1% of the respondents considered cattle trypanosomosis as an economically important cattle disease. It accounted for 64.6% of the total annual deaths with US$200/household financial loss. The respondents also associated the occurrence of the disease with tsetse fly and explained that May and June were peak risk months of the year for the disease and its vectors. Trypanocidals were used at a mean frequency of 5.7 times treatments per animal per year. Local communities were willing to participate and integrate themselves in any forthcoming efforts to minimize the impacts of the disease and its vectors in the area (Seyoum et al., 2011).

1.6.6. Scattered studies in different valleys

Some fragmented cattle trypanosomosis surveys were done in different valleys. A study in Chena district of Kefa zone in southwest Ethiopia revealed that of a total of 391 cattle, 6.9% (27) of the animals were infected by trypanosomes: *T. congolense* (4.89%), *T. vivax* (1.54%), and *T. b. brucei* (0.51%). The prevalence of *T. congolense* and *T. b. brucei* varied significantly. The prevalence in male (7.79%) and female (5.62%) animals was similar whilst it was significantly different in young (2.24%) and adult (8.29%) cattle. The prevalence in good (7.28%), medium (0.78%) and poor (13.39%) body conditioned animals was significantly different. The mean PCV of the infected animals (17.56%) was significantly lower than that of the non-infected animals (25.4%) (Alemayehu et al., 2012).

In Gojeb valley of Gibe (Omo river system at Shebe Sombo woreda) the trypanosome prevalence in dissected flies was 24.5% in *G. pallidipes* (49 of 200) and 22.8% in *G.f. fuscipes* (42 of 184). Out of 200 dissected *G. pallidipes* flies 33(16.5%), 15 (7.5%) and 1(0.5%) were infected by *T. vivax, T. congolense* and *T. brucei*, respectively. On the other hand, out of 184 dissected *G.f. fuscipes*, 27(14.7%) were infected by *T. vivax* and 15 (8.2%) were infected by *T. congolense. T. vivax* is higher in both species of tsetse flies than *T.congolense*. Infection rates in male and female flies of *G. pallidipes* (18.35% vs. 31.87%) and *G.f. fuscipes* (21.43% vs. 24%) did not differ significantly (Bitew et al., 2011).

A survey was conducted in two districts bordering Anger river valley in East Wollega Zone, Western Ethiopia. The prevalence of trypanosomosis was 5.42% in Sibu Sire (vector control implemented area) and 11.88% in Guto Gida (non-control implemented area). The prevalence for trypanosome species was 63.6% and 36% for *T. vivax* and *T. congolense*, respectively. The mean

PCV values of parasitemic (23%) and aparasitemic (26%) animals were significantly different. A total of 1478 flies from both sites was captured using monoconical traps for 72 hours. The highest fly density was found for *G. tachinoides* (6.8 fly/trap/day), followed by *Stomoxys* (5.8 fly/trap/day) and others (0.8 fly/trap/day). The tsetse fly density in the non-controlled dictrict (Guto Gida) was 8.4 flies/trap/day and significantly higher than in the controlled district (Sibu Sire) with 0.2 flies/trap/day (Dagnachew and Shibeshi, 2011).

A cattle trypanosomosis study in Guraferda and Sheko districts of Bench Maji Zone in South Western Ethiopia provided a prevalence of 4.4% of which 36.36% were *T. congolense*, 18.18% *T. vivax* and 9.09% *T.brucei*. *G. pallidipes* and *G.f.fuscipes* were trapped along with Stomoxys and Tabanus. The apparent density of tsetse flies was 2.83 flies per trap per day. The NGU trap caught significantly more *G. pallidipes* while the biconical trap caught more *G. fuscipes* (Tadesse and Tsegaye, 2010).

The prevalence of cattle trypanosomosis was 28.1% (108/384) in Asosa district of Benishangul Gumuz in which older and poor body conditioned animals were significantly more infected. *T. congolense* (72/108, 66.7%) was the predominant species followed by mixed *T. vivax* and *T. congolense* (21/108, 19.4%), *T. vivax* (10/108, 9.3%) and *T. brucei* (5/108, 4.6%) (Mulaw et al., 2011).

1.7. References

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Chapter 2:

General and Specific Objectives

2.1. Aims of the study

African trypanosomosis can be fatal both for human and animal. Besides death, it causes substantial economic losses. Tsetse flies function as the main biological vectors of African trypanosomes. Although a small number of drugs against HAT and AAT are available, drug resistance has developed over the years so that in many locations in Africa, including Ethiopia, drugs have lost their efficiency (Delespaux and de Koning, 2007). Subsequently, the next long lasting economically viable alternative control option is to reduce or eradicate the vector.

Tsetse flies have invaded most of the fertile savannah woodlands and forests of sub-Saharan Africa thus putting a substantial number of animals and humans at risk (Leak, 1999). A sound understanding of tsetse population structure, tsetse behavior, host preference of tsetse for blood meal source, tsetse habitat, distribution and abundance and its temporo-spatial heterogeneity is essential in optimising vector control strategies (Leak, 1999). Tsetse fly prevails only in the west, south-western Ethiopia and to some extent in southern rift-valley. The remaining part of the country is too high (central and north Ethiopia) or too arid (east and south eastern Ethiopia), thus not suitable for tsetse flies. However, the last area-wide tsetse abundance and distribution study has been conducted 30 years ago (Langridge 1976; Fuller, 1978). Since then, only specific studies have taken place. In a first study, the altitudinal heterogeneity in distribution was studied (Tikubet and Gemetchu, 1984) and in a second study invasions of tsetse in areas where tsetse was absent before (Leak et al., 1993) have been reported. It can be assumed, however, that tsetse distribution and abundance has been changed dramatically in the last 30 years given the dynamic modifications in ecology and climate, the shifts in land use, the settlement of huge stocks of insecticide baited livestock, and human population increase in tsetse infested areas (Reid et al., 2000).

As Africa, including Ethiopia, intensifies the fight against tsetse, continuous monitoring of AAT and the vector is essential. Due to the ongoing interventions, both the disease and the vector could decline to a low level beyond the diagnostic capacity of microscopy and fly catching by traps. The current inefficient detection methods for tsetse fly presence and AAT could lead to the false impression that AAT and the vector have been eradicated, which could next lead to quitting the vector control interventions; subsequently the disease risk will rise. Therefore, tools that can quantify the level of exposure of cattle to tsetse earlier are essential to set up a more reliable early warning system. Furthermore, the lack of a reliable marker for exposure of animals to tsetse bite has hindered the fast identification of high risk geographic areas, herds and animals within a herd

that would require immediate intervention thereby deterring the risk of trypanosome transmission.

Another essential epidemiological parameter is the trypanosome infection rate of the vector as it determines the intensity of the transmission of the parasite by the vector. In this regard, the availability of reliable sampling, monitoring and surveillance tools of tsetse flies are fundamental in rapid identification of high risk areas for immediate actions and guiding the control decisions when and where to implement (Habtemariam et al., 1986; Rogers and Williams, 1993).

2.2. General and specific objectives

General objective

- ✓ To evaluate the abundance of tsetse flies, and the trypanosome infection prevalence in the tsetse fly and cattle in Ethiopia.

Specific objectives

- ✓ To update the spatial distribution of tsetse species and trypanosomes in Ethiopia.
- ✓ To develop a diagnostic tool to assess the challenge of animals to tsetse flies based on the antibody response of animals against tsetse fly saliva proteins.
- ✓ To review the trypanosome infection prevalence in tsetse fly populations involved in trypanosome transmission.

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Chapter 3:

Spatial distribution of *Glossina* sp. and *Trypanosoma* sp. in south-western Ethiopia

Adapted from

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3.1. Abstract

Accurate information on the distribution of the tsetse fly is of paramount importance to better control animal trypanosomosis. Entomological and parasitological surveys were conducted in the tsetse belt of south-western Ethiopia to describe the prevalence of trypanosomosis (PoT), the abundance of tsetse flies (AT) and to evaluate the association with potential risk factors. The study was conducted between 2009 and 2012. The parasitological survey data were analysed by a random effects logistic regression model, whereas the entomological survey data were analysed by a Poisson regression model. The percentage of animals with trypanosomosis was regressed on the tsetse fly count using a random effects logistic regression model. The following six risk factors were evaluated for PoT *(i)* altitude: significant and inverse correlation with trypanosomosis, *(ii)* annual variation of PoT: no significant difference between years, *(iii)* regional state: compared to Benishangul-Gumuz (18.0 %), the three remaining regional states showed significantly lower PoT, *(iv)* river system: the PoT differed significantly between the river systems, *(v)* sex: male animals (11.0 %) were more affected than females (9.0 %), and finally *(vi)* age at sampling: no difference between the considered classes. The three trypanosome species observed were *T. congolense* (76.0 %), *T. vivax (18.1 %), T. b. brucei (3.6 %),* and mixed *T. congolense*/*vivax* (2.4 %). The first four risk factors listed above were also evaluated for AT, and all have a significant effect on AT. In the multivariable model only altitude was retained with AT decreasing with increasing altitude. Four different *Glossina* species were identified i.e. *G. tachinoides* (52.0 %), *G. pallidipes* (26.0 %), *G. m. submorsitans* (15.0 %) and *G. f. fuscipes* (7.0 %). Significant differences in catches/trap/day between districts were observed for each species. No association could be found between the tsetse fly counts and trypanosomosis prevalence. Trypanosomosis remains a constraint to livestock production in south-western Ethiopia. Altitude had a significant impact on AT and PoT. PoT is not associated with AT, which could be explained by the importance of mechanical transmission. This needs to be investigated further as it might jeopardize control strategies that target the tsetse fly population.

Keywords: *Trypanosoma*, *Glossina* , Ethiopia, Cattle, Risk factors

3.2. Introduction

In sub-Saharan Africa, trypanosomosis is responsible for poverty, weak economic growth and low agricultural production resulting in subsistence livelihood (Ahmed and Dairri, 1987; Jemal and Hugh-Jones, 1995; Kristjanson et al., 1999; Baumgärtner et al., 2008). In regions under challenge of trypanosomosis, land cannot be exploited for livestock rearing. The resulting lack of draught power is further compromising crop production. In rural Africa, livestock breeding constitutes an alternative banking system and contributes to social wealth and welfare. However, 48 million (=30.0 %) African cattle, not including other livestock species, are exposed to trypanosomosis (Murray and Gray, 1984)

Ethiopia covers an area of 1.1 million km^2 with 240,000 km^2 of fertile areas under threat of trypanosomosis. Particularly affected are the western and southern lowlands, preventing agricultural activities. Drastic droughts in the 70's and early 80's have caused a significant number of people to move from the northern highlands to the tsetse-infested south-western region in search of fertile land (Dejene, 1990). Since the 70's, this situation has worsened by repeated abnormal climatic fluctuations linked to El Niño (Comenetz and Caviedes, 2002). Furthermore, new governmental land use regulations that were adopted between 1987 and 2005 resulted in a threefold increase in utilised agricultural land (Reid et al., 2000; Wood, 1993). This "demographic" clearing, whose effects are comparable to the bush clearing strategy of colonial Africa, substantially changed the distribution and abundance of the different tsetse species, deemed to be the most important vector for trypanosomes (Lee and Maurice, 1983). Currently, 14.8 million cattle, 6.1 million sheep and goats, and 1.2 million equines are at risk of trypanosomosis in this recently settled south-western region. Tsetse control is organised by the Ethiopian government through NICETT (National Institute for the Control and Eradication of Tsetse and Trypanosomosis); formerly represented by STEP (Southern Tsetse Eradication Program). Their strategies comprise the use of insecticidal pour-ons and insecticide impregnated traps and targets. Complementary to those vector control activities, trypanocidal drug treatment remains the most widely used control strategy because it is available and most affordable for livestock breeders. Trypanocides minimize the impact of the parasite on animal health and also reduce the period that the animal is infectious for possible vectors (Slingenbergh, 1992). The presence of single and multiple drug resistant trypanosome strains in different locations (Afewerk et al., 2000; Tewelde et al., 2004; Moti et al., 2012) is hampering the success of chemotherapeutic and prophylactic approaches. For decades, the association of vector control and

chemotherapy has been used to minimize the risk and impact of trypanosomosis (Malele et al., 2011). However, despite more than 30 years of various attempts of tsetse and trypanosomosis control, tsetse flies have expanded their distribution and reinvaded previously tsetse-free areas at a rate of 200 m/year (Vreysen et al., 1999; Tikubet and Gemechu, 1984). This failure in achieving sustainable results is explained through fragmented and uncoordinated actions induced by poor information coupled with the absence of long-term coherent policies (Brightwell et al., 2001). Tsetse populations are highly resilient; populations seem to restore and expand as soon as control measures are discontinued (Rogers and Randolph, 1984). Accurate data on vector and parasite distributions as well as on risk factors for trypanosomosis among the domestic hosts are of paramount importance to control the disease with the ultimate goal of achieving eradication.

Despite the importance of such data, the last country-wide census on tsetse flies and trypanosomosis was conducted by Langridge in 1976. The aim of this study was thus *(i)* to update the available data by conducting large entomological and parasitological surveys and *(ii)* to identify the main risk factors influencing the transmission of the disease.

3.3. Methods

3.3.1. Study region

The study was conducted in south-western Ethiopia. The parasitological survey was conducted in a region located between 7°12′ to 10°09′N latitude and 34°39′ to 35°00’E longitude and the entomological survey between 8°22′ to 12°13′N latitude and 35°32′ to 37°28′E longitude (Figure 3.1 and 3. 2). The altitudes of the study regions ranged between 1040 and 2012 m and between 628 and 1673 m.a.s.l. (metres above sea level) for the parasitological and the entomological surveys, respectively. The region contains a hydrographic network including the Abay (Blue Nile), Didessa, Anger, Baro Akobo, Birbir, Dabus, Ghibe and Tekeze rivers and their tributaries. The Abay Didessa has a runoff of 52.6 billion m^3/year and covers 199,812 km^2 catchment area with elevation ranging from 500 to 4261 m. The Boro-Akobo has 23.6 billion m^3/year runoff and covers 75,912 km^2 catchment area with elevation ranging from 390 to 3244 m. The Ghibe-Omo river has 17.9 billion m3/year runoff, 79,000 km2 catchment area and a variety of wildlife parks (Awulachew et al., 2011). There are two rainy seasons: the short one from the end of February to the end of April and the long one from June to September. The current study area includes four of the ten ecosystems of the country i.e. *(i)* woodland, *(ii)* lowland tropical forest, *(iii)* montane moist forest and *(iv)* montane dry evergreen (IBC, 2007). The primary forests in these ecosystems

together with the river networks constitute sanctuaries for tsetse flies and diverse wildlife (Abebe, 2010) despite an intense deforestation rate. Firewood and land to feed the growing population are the main causes of encroachment (Reusing, 2000). Livestock is mainly fed on crop residues (at the end of the rainy season, beginning of the dry season) and on naturally persistent pastures. As most of the land is intensively cultivated during the long rainy season, cattle are moved to the vicinity of the forests where permanent grassland is available to avoid damage to the crops. Cattle are the dominant livestock species. The Horro breed is concentrated in the southwest and west of Ethiopia, the Abigar (Nuer) breed in the Gambela region, the Gurage breed in the Gurage zone and the Fogera breed in northwest Ethiopia in Amhara region (Lemecha et al., 2006).

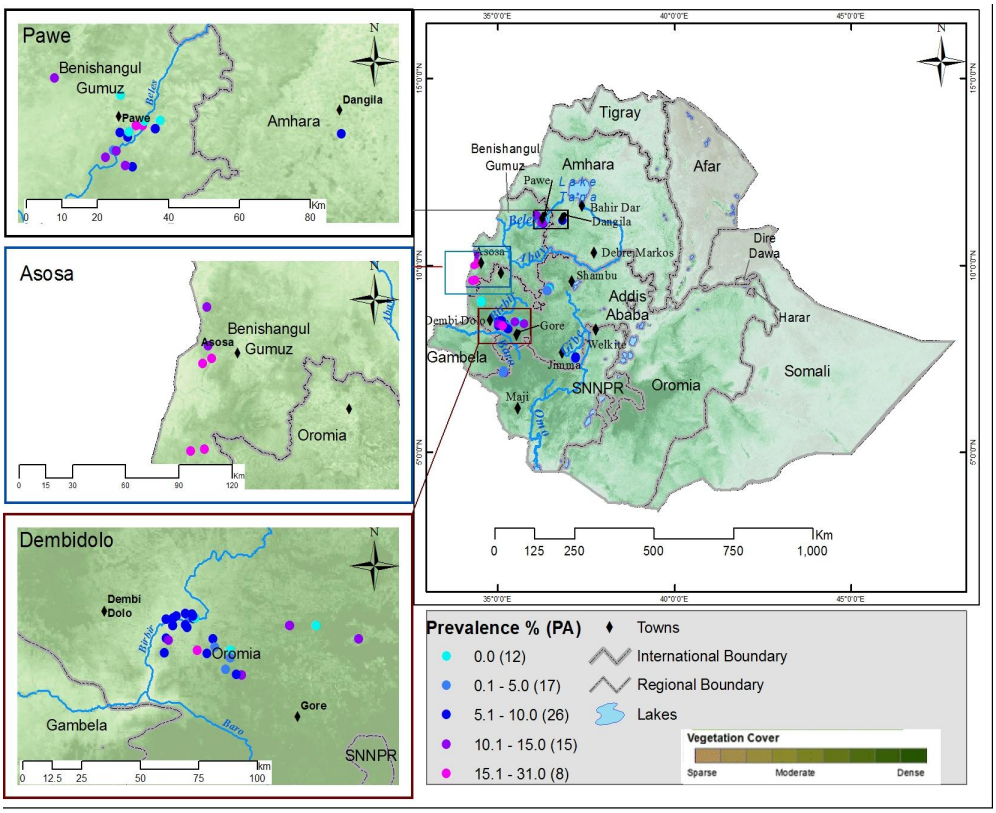

Figure 3.1. Map of the study area showing the selected peasant associations, trypanosome prevalence (%) and distribution. The overall prevalence of cattle trypanosomosis over the 82 selected PAs is 9.6% with 0% in 12 PAs, 0.1-5% in 17 PAs, 5.1-10% in 28 PAs, 10.1-15% in 15 PAs and 15.1-31% in 8 PAs indicating substantial variation between the PAs.

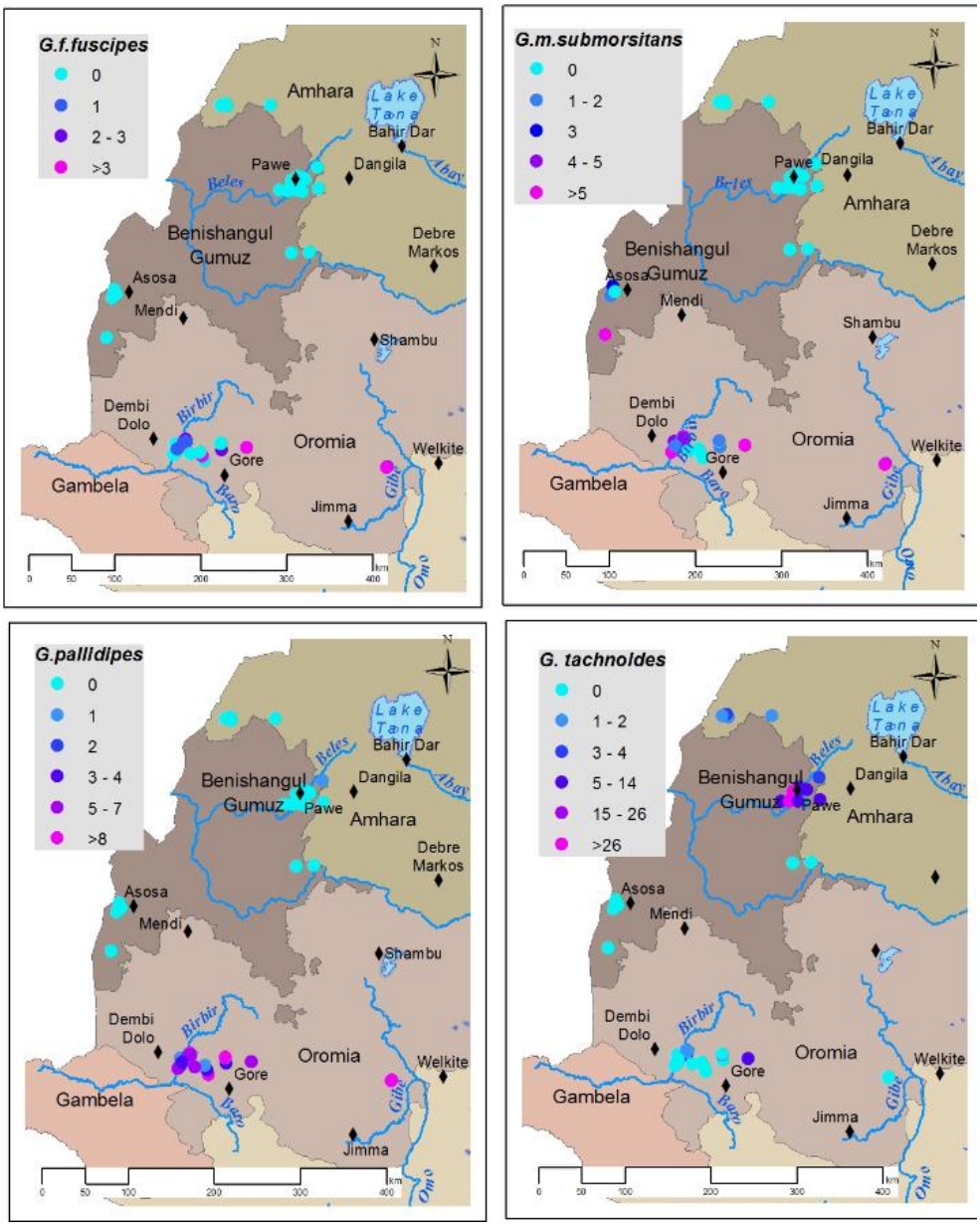

Figure 3.2.Tsetse fly catches per trap per day and distribution in the studied area of Ethiopia. Four tsetse fly species (*G. pallidipes*, *G .m. submorsitans*, *G. fuscipes* and *G. tachnoides*) were detected (only *G. tachnoides* in Amhara region and *G. m. submorsitans* in Benishangul-Gumuz region whilst more than one tsetse fly species was observed in Oromia region).

Trypanosomosis occurs in five of the nine Ethiopian regional states (Oromia, Amhara, Gambela, Benishangul-Gumuz and Southern Nations Nationalities and Peoples Region) comprising 240,000 km^2 of land area. Sampling was only carried out in 4 states (excluding Southern Nations Nationalities and Peoples Region). From the 4 regional states, eleven districts were purposively selected based on the burden of trypanosomosis reported by farmers and based on the expert opinion of local veterinarians. Local experts used financial cost, morbidity, mortality and withdrawal from draft power as criteria to estimate the trypanosomosis burden. The 11 districts comprise a total of 340 peasant associations (PA). A PA is the lowest administrative structure in Ethiopia with an average land area of 53 km^2. The tributaries in the vicinity of each PA (within a 3.5 km radius) were allocated to one of the four main river systems, i.e., Ghibe, Baro-Akobo, Abay (Blue Nile) and Abay-Didessa.

3.3.2. Sampling frame

Two-hundred PAs served as sampling frame since 140 had to be excluded from the study due to poor accessibility.

A total of 30 PAs (Figure 3.1) were selected at random from the accessible 200 PAs, and both a parasitological and entomological survey was carried out in those 30 PAs. In another set of 52 randomly selected PAs, only a parasitological survey was done, and in another set of eight randomly selected PAs, only an entomological survey was carried out. Therefore, an entomological survey was conducted in 38 PAs in seven of the above selected 11 districts. Additionally, only an entomological survey was done in 14 PAs that were randomly selected from 92 PAs in 3 districts (which were not a subset of the above 11 districts); thus entomological surveys were done in a total of 52 PAs in 10 districts in this study. These 3 districts (Quara, Chora Botor and Darimu) were purposively included. Quara district in north Amhara region, Chora Botor district in Oromia bordering Ghibe valley and Shewa highlands and Darimu district in Oromia bordering Birbir valley and Ilu-Abaabora highlands that have been reported to be the limit of tsetse fly distribution as per the information from Zonal Veterinary offices.

Within each PA selected for parasitological survey, a minimum of 75 heads of cattle were selected at random and sampled, which led to a total of 7021 animals from those 82 PAs in 11 districts (on average 85 heads of cattle/PA).

3.3.3. Parasitological survey

Between 2009 and 2012, blood samples were collected from these 7021 animals. The geographical reference of the PA, the number of animals sampled per PA and their age (based on the teeth method three age classes were defined: >4, >2 to ≤4 and ≤2 years), sex and trypanosome infection status were recorded. Sampling took place during the 5 months of the dry season (October to February) and the 3 months of the short rainy season (March to May), but each PA was only sampled once. Blood was taken from the ear vein, collected in a heparinized capillary tube and centrifuged by the Micro-Haematocrit Centrifugation Technique (Woo, 1970). A minimum of 50 fields were examined using a standard light microscopy at 400x magnification. Trypanosomes were identified according to their movement pattern and later confirmed by the examination of Giemsa stained thin smears (Luckins, 1992).

The experimental protocol and sampling were approved by the Ethical Committee of Addis Ababa University and the Zonal Agricultural Bureau. The background of the study was explained to the Peasant Associations and consent of the farmers was asked for sampling of their animals.

3.3.4. Entomological survey

The entomological survey was conducted between 2009-2011 in 52 PAs at the same time as parasitological survey. In 30 PAs (out of 52) parasitological and entomological samplings were conducted at the same time. A total of 1046 mono-pyramidal traps (Gouteux et al., 1986) were used throughout in the 52 PAs. This trap was chosen as it was reported to be cheaper (US$ 2.61) and required less material to construct than pyramidal trap (US$ 3.48), biconical and the modified pyramidal traps (US$ 4.06 each) (Abila et al., 2007). Moreover, this is a standard trap available for Ethiopian conditions (Slingenbergh, 1992). Three tsetse fly attractants (acetone, octenol and cow urine) were used (Dean et al., 1969; Vale, 1974). Traps were deployed for 2 days and flies were collected twice a day at around 10:00 AM and 6:00 PM. Tsetse flies were counted, species and sex were determined. The number of tsetse flies captured per trap per day was used as indicator of the AT.

3.3.5. Data analysis

The relationship between the presence of trypanosomes and the potential risk factors of altitude, sampling year, regional state, river system, sex and age was modelled by a random effects logistic regression model with PA incorporated as random effect (Vanleeuwen et al., 2010). First,

each potential risk factor was included alone in the model (univariate analysis), next, potential risk factors with a p-value lower than 0.10 in the univariate analysis were incorporated jointly (Hosmer et al., 2013), together with their two-way interactions (multivariate analysis), and the forward selection procedure was applied to obtain the final model (Dohoo et al., 2003). The odds ratio with its 95 % confidence interval was used as summary statistic.

The AT of tsetse flies in each PA was given by the number of flies captured in all deployed traps. The relationship between the AT in a PA and the potential risk factors of altitude, sampling year, regional state and river system was modelled by a Poisson regression model with the number of traps in the PA included as fixed offset term (McCullagh and Nelder, 1989). First, each potential risk factor was included alone in the model (univariate analysis), next potential risk factors with a p-value lower than 0.10 in the univariate analysis were incorporated jointly, together with their two-way interactions (multivariate analysis), and the forward selection procedure was applied to obtain the final model. The incidence rate ratio (IRR) with its 95 % confidence interval was used as summary statistic.

The distribution of the ATs over the four different species was compared between the districts using the Kruskall-Wallis test. The difference of the total number of tsetse flies of the two sexes within each trap was compared for each *Glossina* species separately using the Wilcoxon rank sum test stratified for trap.

Finally, the relationship between trypanosome presence and AT was investigated by a random effect logistic regression model with PA incorporated as random effect, the trypanosome status of the animal as response variable and the AT in the PA as risk factor.

3.4. Results

3.4.1. Trypanosomosis prevalence, distribution and risk factors

Out of 7021 examined cattle, 675 (9.61 %) were trypanosome-positive. The predominant species was *T. congolense* (76.0 %), followed by *T. vivax* (18.1 %), *T. b. brucei* (3.6 %), and mixed *T. congolense*/*vivax* infections (2.4 %). Trypanosomosis prevalence varied significantly between the PAs. In 14.6 % of the PAs ($n = 12$), no trypanosomes were detected. The results are summarized in Figure 3.1. When compared to Benishangul-Gumuz (18.0 %), the 3 other regional states had a significantly lower trypanosomosis burden: Amhara (12.0 %), Oromia (6.0 %) and Gambela (5.0 %).

In the univariate model (Table 3.1), low altitude was significantly and inversely associated with trypanosomosis. Geographical regions with an altitude lower or equal to 1200 m showed the highest trypanosomosis prevalence (12.0 %). Annual variation of the trypanosomosis burden was not significant between years. The highest and lowest trypanosomosis prevalence was observed at the first year (11.0 %) and the last year (4.0 %), respectively. Male animals (11.0 %) were significantly more often affected than females (9 %, $p = 0.01$). The distribution of trypanosomosis also varied significantly between the different river systems: the prevalence was correlated with the size of the river system with 5.0 % around the Ghibe river (17.9 billion m^3/year), 7.0 % around the Boro-Akobo (23.6 billion m^3/year), 11.0 % around the Abay/Blue Nile and 15.0 % around the enlarged Abay Didessa (52.6 billion m^3/year) (Awulachew et al., 2011). The different age classes did not differ significantly with respect to trypanosomosis burden. The highest and lowest trypanosomosis prevalence was observed in cattle of age > 4 years (11.0 %) and ≤ 2 years (6 %), respectively. In the final multivariable model, only altitude and sex had a significant effect (Table 3.2) confirming the trend of the univariate analysis.

3.4.2. Distribution, abundance and risk factors of Tsetse flies

A total of 14,698 tsetse flies were trapped. Four different species were identified i.e. *G. tachinoides* (52 %), *G. pallidipes* (26 %), *G. m. submorsitans* (15 %) and *G. fuscipes fuscipes* (7 %). Significant differences in AT between districts were observed globally and for each of the *Glossina* species separately ($p < 0.0001$). The mean figure for AT was 7.45 catches/trap/day but considerable differences between PAs were observed.

No catches were recorded at four PAs, i.e. Awjeben (Qwara district), Silga 22 (Asosa district), Tsiwuli and Yimale (Guangua district) and up to 78 catches/trap/day in the Pawe district. The AT and distribution across sampled PAs is shown in Figure 3.2. *G. tachinoides* was the sole species trapped in the PAs of Guangua, Jawi, Pawe and Qwara districts. In the PAs of Asosa district, only *G. m. submorsitans* was trapped whilst all four tsetse species were detected in the PAs of Dalesadi and Dalewabara. Two species were observed in the PAs of Darimu district (*pallidipes* and *fuscipes*), of Hawagelan district (*pallidipes* and *submorsitans*), whereas in Choraboter district three species (*submorsitans*, *pallidipes* and *fuscipes*) were observed. More female than male tsetse were trapped with 53 % and 47 % respectively ($p = 0.01$). This was also the case for each species considered individually (Table 3.3).

Table 3.1. Distribution of cattle trypanosomosis among potential risk factors and univariate analysis

Risk factor	Category	N (N+)	Prevalence	95 % CI	p-value
Altitude	≤1200 m	971 (118)	12.2 %	10.2, 14.4	0.0002
	>1200 m, ≤1300 m	518 (43)	8.3 %	6.1, 11.0	
	>1300 m, ≤1400 m	910 (65)	7.1 %	5.6, 9.0	
	>1400 m, ≤1500 m	2936 (334)	11.4 %	10.2, 12.6	
	>1500 m, ≤1600 m	1074 (86)	8.0 %	6.5, 9.8	
	>1600 m	612 (29)	4.7 %	3.2, 6.7	
Year	2009	3191 (345)	10.8 %	9.8, 11.9	0.2154
	2010	2619 (255)	9.7 %	8.6, 10.9	
	2011	1054 (68)	6.5 %	5.0, 8.1	
	2012	157 (7)	4.5 %	0.18, 9.0	
Regional state	Benshangul-Gumuz	1763(312)	17.7 %	15.9, 19.6	0.0002
	Amhara	552 (65)	11.8 %	9.2, 14.8	
	Oromia	4214 (271)	6.4 %	5.7, 7.2	
	Gambela	492 (27)	5.5 %	3.6, 7.9	
Sex	Male	3356 (355)	10.6 %	9.6, 11.7	0.0102
	Female	3665 (320)	8.7 %	7.8, 9.7	
River system	Abay Didessa	1688(256)	15.2 %	13.5, 17.0	0.0419
	Abay	1294 (145)	11.2 %	9.5, 13.1	
	Baro Akobo	3377 (240)	7.1 %	6.3, 8.0	
	Ghibe	662 (34)	5.1 %	3.6, 7.1	
Age (years)	>4	4096 (435)	10.6 %	9.7, 11.6	0.2211
	>2, ≤4	2321 (201)	8.7 %	7.5, 9.9	
	≤2	604 (39)	6.5 %	4.6, 8.7	

With *N* number sampled, *(N+)* number positive, *CI* Confidence Interval

Table 3.2. The multivariable model presenting the risk factors associated with trypanosomosis in Ethiopia

Risk factor	Category	Odds ratio	95 % CI	p-value
Altitude	≤1200 m[a]	1	-	-
	>1200 m, ≤1300 m	0.27	(0.14, 0.51)	<0.001
	>1300 m, ≤1400 m	0.40	(0.20, 0.80)	0.009
	>1400 m, ≤1500 m	0.53	(0.32, 0.90)	0.019
	>1500 m, ≤1600 m	0.32	(0.15, 0.65)	0.002
	>1600 m	0.34	(0.15, 0.74)	0.007
Sex	Female[a]	1	-	-
	Male	1.25	(1.06, 1.49)	0.01

With *CI* Confidence Interval, [a] reference

Table 3.3. Distribution of tsetse flies by species, sex and district

District	n.s.	*G. m. submorsitans*			*G. pallidipes*			*G. f. fuscipes*			*G. tachinoides*			
		M	**F**	∑	**M**	**F**	∑	**M**	**F**	∑	**M**	**F**	∑	∑∑
Dalesadi	4	230	456	686	337	700	1037	78	151	229	74	74	148	2100
Dalewabera	4	197	320	517	198	388	586	147	220	367	163	207	370	1840
Choraboter	3	389	281	670	777	329	1106	207	150	357	-	-	-	2133
Hawagelan	3	69	121	190	127	247	374	1	6	7	-	-	-	571
Darimu	2	-	-	-	185	483	668	49	62	111	-	-	-	779
Asosa	1	41	93	134	-	-	-	-	-	-	-	-	-	134
Guangua	1	-	-	-	-	-	-	-	-	-	226	210	436	436
Jawi	1	-	-	-	-	-	-	-	-	-	341	350	691	691
Pawe	1	-	-	-	-	-	-	-	-	-	3081	2762	5843	5843
Qwara	1	-	-	-	-	-	-	-	-	-	62	109	171	171
∑		926	1271	2197	1624	2147	3771	482	589	1071	3947	3712	7659	14698
p-value		$p < 0.001$			$p < 0.001$			$p = 0.047$			$p = 0.577$			

With *n.s* number of species, *M* Male, *F* Female, ∑ = Total, ∑∑ = Grand total, p-values refer to comparison between sexes

In the univariate model (Table 3.4), all risk factors had a significant effect on AT, but only altitude was retained in the multivariable analysis (Table 3.5). Low altitude was significantly and positively associated with AT. Geographical regions with an altitude lower or equal to 1200 m showed the highest average count of 17.1 catches/trap/day. Astonishingly, geographical regions above 1500 m showed an unexpected high average count of 12. This is linked to one single PA (Abuna Gali) where the AT was abnormally high. Finally, no relationship between AT and trypanosomosis prevalence was found (OR = 1, 95 % CI: [0.99;1], $p = 0.348$).

Table 3.4. Average tsetse fly counts among potential risk factors and univariate analysis

Risk factor	Category	Average count	95 % CI	p-value
Altitude	≤1200 m[a]	17.1	16.6,17.6	<0.001
	>1200 m, ≤1300 m	12.0	11.6,12.5	
	>1300 m, ≤1400 m	13.6	13.2,14.0	
	>1400 m, ≤1500 m	5.9	5.5,6.2	
	>1500 m	12.0	10.9,13.2	
Year	2009	10.4	10.1,10.7	<0.001
	2010	18.3	17.9,18.7	
	2011	6.0	5.7,6.4	
Regional state	Benshangul-Gumuz	32.7	31.8,33.6	<0.001
	Amhara	3.3	3.1,3.5	
	Oromia	14.5	14.1,14.8	
River system	Abay Didessa	2.9	2.5,3.5	<0.001
	Abay	12.3	12.0,12.7	
	Baro Akobo	14.5	14.1,14.8	

Table 3.5. The multivariable model presenting the risk factors associated with tsetse fly abundance in Ethiopia

Risk factor	Category	IRR	95 % CI	p-value
Altitude	≤1200 m[a]	1	-	-
	>1200 m, ≤1300 m	0.71	(0.67,0.74)	<0.001
	>1300 m, ≤1400 m	0.79	(0.76, 0.83)	<0.001
	>1400 m, ≤1500 m	0.34	(0.32,0.37)	<0.001
	>1500 m	0.70	(0.63,0.78)	<0.001

With *IRR* Incidence Rate Ratio, *CI* Confidence Interval, [a] reference

3.5. Discussion

3.5.1. Trypanosomosis

The overall prevalence of trypanosomosis was 9.6 %. Using the same parasitological technique for diagnosis, a comparable prevalence was reported in north-western Ethiopia at Jawi district (Mekuria and Gadissa, 2011) and the western Oromia regional state (Tasew and Duguma, 2012). Prevalence in the PAs ranged from 1.1 % to 30.8 %. *T. congolense* was the most predominant species (76.0 %) followed by *T. vivax* (18.1 %) and *T. brucei* (3.6 %). This is in agreement with the observations made by other groups in different tsetse-infested areas in Ethiopia (Langride, 1976; Abebe and Jobre, 1996; Afewerk et al., 2000; Mekuria and Gadissa, 2011; Tasew and Duguma, 2012).

Altitude is a well-known limiting factor for cattle trypanosomosis. The altitudinal difference in trypanosomosis burden is most often explained by the significant variation in tsetse densities across altitudes (Leak, 1999), but this point will be discussed later when describing the relationship between trypanosomosis and AT.

The annual variation in trypanosomosis prevalence was not significant from one year to another. However, the trend goes towards a decrease in prevalence, which is in line with the growing intensity of human encroachment and the resulting land use changes. It might also be explained by the intensity of control by NICETT but we do not have data to perform the analysis to substantiate it.

The highest prevalence of trypanosomosis was observed in PAs of the Asosa district where one single species of *Glossina* was trapped (*G. m. submorsitans*). This can be explained by the higher susceptibility of the *morsitans* group to infections by *T. congolense* (Reifenberg et al., 1997). In contrast, the lowest prevalence rates were observed in PAs of Dalesadi and Dalewabara districts where four species of *Glossina* were trapped. In 14.6 % of the PAs ($n = 12$), no trypanosomes were detected (Figure 3.1). From our data, except for the higher susceptibility of the *morsitans* group to trypanosome infections, no clear explanation could be given for those variations (e.g. land cover, consistent link to a particular vector species). However, looking at the literature some common factors are associated with prevalence variability: annual and seasonal differences in sampling time, cattle abundance at watering places, micro-environmental ecological conditions (Leak et al., 1990; Specht, 2008; Hoppenheit et al., 2010), variations in tsetse and other hematophagous fly (mainly *Stomoxis* spp. and tabanids) densities and their respective vectorial

capacity and seasonality (Ahmed et al., 2006; Hoppenheit et al., 2013; Tchouomene-Labou et al., 2013) and the trypanotolerance/susceptibility of local cattle breeds (Leak et al., 1990; Lemecha et al., 2006; Stein et al., 2011).

The susceptibility of male cattle to trypanosome infections was higher than that of females. This can be explained by *(i)* their higher attractiveness for flies, *(ii)* a higher stress linked to their use as draft animals and *(iii)* the malnourishment of young males used prematurely for traction work with an insufficient food supply. Similar situations were reported in Ethiopia and Zambia (Conner, 1994; Simukoko et al., 2007; Tasew and Duguma, 2012).

3.5.2. *Glossina*

A total of 14,698 tsetse flies were trapped. Four different species were identified i.e. *G. tachinoides* (52 %), *G. pallidipes* (26 %), *G. morsitansmorsitans submorsitans* (15 %) and *G. fuscipes fuscipes* (7 %). Significant differences in AT were observed between districts globally and for each of the *Glossina* species separately.

Qualitatively, Dale Sadi and Dale Wabera districts were colonized by four *Glossina* species whilst one, two or three species prevailed in the remaining 8 districts (Table 3.5). In Asosa district, only *G. m. submorsitans* was observed, which is probably due to the fact that Asosa is characterized by an unspoiled savannah environment, as this species is particularly sensitive to encroachment. Additionally this area is not drained by large rivers, which explains the absence of riverine flies. In the other districts, ecological features are more diverse, providing niches for different coexisting species. A number of reports have shown that the overlapping of two to three tsetse species is common (Gouteux and Jarry, 1998; Malele et al., 2007). *G. pallidipes*, *G. m. submorsitans*, *G. tachinoides* and *G. f. fuscipes* were heterogeneously distributed in the western and north western zones of Ethiopia which is in agreement with previous studies (Tikubet and Gemetchu, 1984; Vreysen et al., 1999).

Differences in the flies' sex ratios were noticed for *G. m. submorsitans* (1 male to 1.37 females), *G. pallidipes* (1 male to 1.32 females) and *G. f. fuscipes* (1 male to 1.22 females). Females are over-represented by 22 to 37 % for each species. This is in agreement with the reports of Leak in 1999 who reported that females would represent 70 % to 80 % in unbiased sampling. A number of causal factors for the sex ratio distortion in favour of females include *(i)* selective attractiveness of the trap (Rowlands et al., 1993; Leak, 1999), *(ii)* a longer life expectancy of females compared to males (Buxton, 1961), *(iii)* male reproducers transmitting X-bearing or a

non-functional Y-bearing sperm (Rawlings and Maudlin, 1984) and *(iv)* a higher mortality rate of the males because of their higher susceptibility to insecticides (Burnett, 1961).

Quantitatively, the AT varied from PA to PA. Indeed, different ecological contexts are obviously translated by different fly densities and species compositions (Slingenbergh, 1992). Low altitude was significantly and positively associated with AT. Indeed, altitude directly influences ecological parameters such as vegetation cover and structure inducing specific microclimatic zones at different elevations (Leak, 1999; IBC, 2007). Additionally, lower temperature at night limits the pupal development and scarcer vegetation renders the environment more stressful for tsetse flies. In Nigeria an odds ratio of 0.91 per 50 m altitude increase was observed with an upper limit of 1800 m a.s.l. above which no tsetse flies were observed (Majekodunmi et al., 2013; Jordan, 1986). Our data confirms this observation except for the sole PA of Abuna Gali were high catches were observed despite the altitude being above 1500 m. The data of this sole PA is pulling the entire category upwards. The most likely explanation for this, is a vegetation particularly dense for this altitude and the proximity of a permanent river that is connected by small seasonal tributaries to the river system of the Didessa wildlife reserve situated 80 km East from Abuna Gali.

Altitude, presence or absence of rivers, trees and wildlife are certainly determining factors for fly abundance but one of the main factors that remains is human encroachment. Indeed, tsetse and human population densities are negatively correlated and tsetse fail to survive in areas inhabited by more than 40 people per km^2 (Ford, 1986). This is particularly true for *G. m. submorsitans*, which is very sensitive to habitat degradation. The varying intensity of tsetse control in each region can somewhat contribute to the observed variations. Unfortunately, no data was available to perform the analysis to substantiate this possibility.

Despite many years spent on tsetse and trypanosomosis control, the problem persists due to mismanagement of available disease control means particularly in terms of geographical distribution and choice of control measures (Jemal and Hugh-Jones, 1995).

3.5.3. Relationship between tsetse abundance and trypanosome prevalence

No relationship between AT and trypanosomosis prevalence was found (Odds Ratio = 1, 95 % CI: [0.99; 1], $p = 0.348$) in the 30 PAs where the comparison was made. This is mainly due to the fact that at low densities the trypanosomosis prevalence varied a lot, from 0 to very high levels. This might be partially explained by an increased vectorial capacity of flies under adverse

conditions (Akoda et al., 2009). Indeed, starvation and high ambient temperatures increase the susceptibility of tsetse flies to trypanosome infections (Kubi et al., 2006; Bouyer et al., 2013), hence increasing the rate of transmission with subsequently increased trypanosomosis prevalence in animals. Another factor that should not be immediately discarded is the possibility of mechanical transmission of trypanosomes by hematophagous flies as previously described for *T. vivax* and *T. congolense* (Desquesnes and Dia, 2003 a & b and 2004) and more specifically in Ethiopia (Roeder et al., 1984; Cherent et al., 2006; Sinshaw et al., 2006; Fikru et al., 2012). However, in our study, the tsetse abundance was not adjusted for the tsetse infection rate rendering the estimation of the real tsetse challenge impossible and consequently, the link between the tsetse challenge and AAT prevalence (Dicko et al., 2015). Further experimental data is thus needed to know if a reduction in tsetse challenge would lead to a significant reduction in trypanosomosis prevalence (Leak et al., 1990). Furthermore, the link between AT and trypanosomosis prevalence is weakened by the fact that cattle bred in PAs located far from rivers and/or suitable tsetse habitats, travel longer distances to reach watering points and grasslands in the dry season: cattle is being brought to the vectors. During the rainy season, water and shade are widespread; cattle are walking shorter distances but flies are more dispersed in this tsetse-favouring environment. The vector host interface is thus facilitated.

3.6. Conclusions

The present study provides a recent update on AT and on trypanosomosis prevalence in the southwest of Ethiopia together with the associated risk factors. Despite increasing human encroachment and vector/disease control operations tsetse are still abundant in the studied region and trypanosomosis remains an impediment to livestock health and production. The high trypanosomosis prevalence with low or nil ATs should be further investigated so that coordinated control strategies can be established.

3.7. Acknowledgement

We would like to thank the Agricultural Bureau of the four Regional States and the Bedele National Trypanosomosis and Tsetse Control Centre for their support during the field work. The VLIR-UOS is also acknowledged for financial support for the whole trypanosomosis project in the framework of the Institutional University Cooperation – Jimma University (IUC-JU) project ZIUS2014AP014.

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Chapter 4:

Serological responses and biomarker evaluation in mice and pigs exposed to tsetse fly bites

Adapted from

Caljon, G.*, Duguma, R.*, De Deken, R., Schauvliege, S., Gasthuys, F., Duchateau, L. and Van Den Abbeele, J., 2014. Serological responses and biomarker evaluation in mice and pigs exposed to tsetse fly bites. *PLoS neglected tropical diseases*, 8(5), e2911. doi:10.1371/journal.pntd.0002911

* Equal contribution

4.1. Abstract

Tsetse flies are obligate blood-feeding insects that transmit African trypanosomes responsible for human sleeping sickness and *nagana* in livestock. The tsetse salivary proteome contains a highly immunogenic family of the endonuclease-like Tsal proteins. In this study, a recombinant version of Tsal1 (rTsal1) was evaluated in an indirect ELISA to quantify the contact with total *Glossina morsitans morsitans* saliva, and thus the tsetse fly bite exposure. Mice and pigs were experimentally exposed to different *G. m. morsitans* exposure regimens, followed by a long-term follow-up of the specific antibody responses against total tsetse fly saliva and rTsal1. In mice, a single tsetse fly bite was sufficient to induce detectable IgG antibody responses with an estimated half-life of 36–40 days. Specific antibody responses could be detected for more than a year after initial exposure, and a single bite was sufficient to boost anti-saliva immunity. Also, plasma collected from tsetse-exposed pigs displayed increased anti-rTsal1 and anti-saliva IgG levels that correlated with the exposure intensity. A strong correlation between the detection of anti-rTsal1 and anti-saliva responses was recorded. The ELISA test performance and intra-laboratory repeatability was adequate in the two tested animal models. Cross-reactivity of the mouse IgGs induced by exposure to different *Glossina* species (*G. m. morsitans*, *G. pallidipes*, *G. palpalis gambiensis* and *G. fuscipes*) and other hematophagous insects (*Stomoxys calcitrans* and *Tabanus yao*) was evaluated. This study illustrates the potential use of rTsal1 from *G. m. morsitans* as a sensitive biomarker of exposure to a broad range of *Glossina* species. We propose that the detection of anti-rTsal1 IgGs could be a promising serological indicator of tsetse fly presence that will be a valuable tool to monitor the impact of tsetse control efforts on the African continent.

4.2. Introduction

Tsetse flies (*Glossina* spp.) are notorious transmitters of trypanosome parasites responsible for Human and Animal African Trypanosomiasis (HAT and AAT). Since 2009, the annual number of reported cases of HAT has dropped below 10,000 (www.who.int; Simarro et al., 2010) with the prospect and challenge of entering into the elimination phase of HAT in the near future (Simarro et al., 2008; Aksoy, 2011). Additionally, some 46 million cattle in sub-Saharan Africa are estimated to be at risk of contracting AAT making deep inroads in the socio-economical development of this continent (Cattand, 2010). Beside active HAT case detection and treatment of humans as well as prophylactic and curative treatment of animals with trypanocidal drugs, tsetse vector control represents an important component of trypanosomiasis control, which is mainly based on the use of insecticides through the sequential aerosol spraying technique (SAT), ground spraying, insecticide-treated targets or insecticide-treated animals [reviewed in (Torr et al., 2005; Schofield and Kabayo, 2008; Vreysen et al., 2013). After a successful campaign as part of an area-wide integrated pest management on Unguja island (Zanzibar) (Vreysen et al., 2000), the sterile insect technique has been added to the vector control arsenal, with ongoing activities in Ethiopia, Senegal and Burkina Faso (Sow et al., 2012) under the auspices of the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC). However, beside laborious conventional entomological surveys, no sensitive rapid tests are yet available to provide a semi-quantitative measure of the evolution of tsetse fly densities in areas subjected to tsetse control interventions. Indeed, easy-to-use monitoring of tsetse fly exposure on a regular basis would be a highly valuable tool in the follow-up of the efficacy of the applied and/or ongoing tsetse fly control activities.

The obligatory blood feeding tsetse flies are the cyclical insect vectors of HAT and a majority of AAT infections are initiated by the bite of an infected tsetse fly. Although it can be assumed that all tsetse fly species could act as vector, a number of *Glossina* species of the *Palpalis* group (e.g. *G. palpalis* spp., *G. fuscipes* spp., *G. tachinoides*) and the *Morsitans* group (e.g. *G. morsitans morsitans*, *G. pallidipes*, *G. swynnertoni*) are implicated as major vectors for HAT and animal trypanosomiasis. Given that only a limited percentage of these tsetse flies acquire an infection with trypanosomes, vertebrate hosts living in the tsetse fly belt are predominantly exposed to the bites of uninfected flies. It has been demonstrated for a number of hematophagous insects that salivary proteins induce humoral immune responses that could represent attractive sero-epidemiological markers of exposure (reviewed in Fontaine et al., 2011). The saliva of *G. m.*

morsitans tsetse flies was documented to contain over 200 protein constituents (Alves-Silva et al., 2010) from which some are implicated in manipulating the vertebrate hemostatic and inflammatory reactions (Caljon et al., 2006a; Caljon et al., 2010; Cappello et al., 1996). In *G. m. morsitans* saliva, the most abundant proteins were shown to be highly immunogenic and to correspond to the 43–45 kDa tsetse salivary gland (Tsal) protein family (Caljon et al., 2006b). The physiological role of these proteins remains elusive, but biochemical characterization revealed that they are nucleic acid binding proteins with low endonuclease activity (Caljon et al., 2012). Immunoglobulin responses to tsetse fly saliva have been detected in humans living in Uganda (Caljon et al., 2006b), Democratic Republic of Congo (Poinsignon et al., 2007 and 2008a) and Guinea (Dama et al., 2013a). Also cattle experimentally exposed to tsetse fly bites displayed elevated levels of anti-saliva antibodies (Somda et al., 2013). Immunoblotting studies using the immune plasma have shown that salivary proteins of several tsetse fly species are recognized by the circulating antibodies (Caljon et al., 2006b; Poinsignon et al., 2007; Dama et al., 2013a). The highly abundant Tsal proteins were commonly recognized by the human plasma and an indirect ELISA using recombinant Tsal1 and Tsal2 proteins as antigens was clearly able to differentiate the tsetse-exposed Ugandan plasma from control plasma (Caljon et al., 2006b). Recently, a peptide (amino acids 18–43) derived from the *G. m. morsitans* adenosine deaminase-related TSGF1 protein was evaluated using a panel of human plasma from West Africa, revealing that obtained ELISA signals correlated with the anticipated levels of tsetse exposure of the tested populations (Dama et al., 2013b). Allergic and anaphylactic reactions against tsetse fly bites have also been reported, in which IgE antibodies directed against an Antigen5-related salivary allergen are implicated (Ellis et al., 1986; Stevens et al., 1996; Caljon et al., 2009). Efforts to develop a serological test based on a TAg5-derived peptide were not yet successful (Dama et al., 2013b).

We here provide experimental evidence that anti-rTsal1 and anti-*G. m. morsitans* saliva antibodies can mark exposure of mice and pigs to tsetse flies. Although the anti-tsetse saliva ELISA exhibits a better average test performance, we propose that a serological assay based on the individual recombinant Tsal1 protein could be an alternative to assess the exposure of populations or herds to tsetse fly challenge and hence could be used for tsetse fly epidemiological studies, for prioritizing tsetse fly control, for monitoring and evaluating tsetse fly control schemes and for risk assessment of trypanosome transmission in endemic regions.

4.3. Methods

4.3.1. Ethics statement

The experiments, maintenance and care of animals complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS n° 123). Rodent care and experimental procedures were performed under approval from the Animal Ethical Committee of the Institute of Tropical Medicine (Permit Nrs. PAR013-MC-M-Tryp and PAR014-MC-K-Tryp). Breeding and experimental work with tsetse flies was approved by the Scientific Institute Public Health department Biosafety and Biotechnology (SBB 219.2007/1410). Pig experiments were approved by the ITM Animal Ethical Committee (Permit Nr. PAR-021) and the Ethical Committee of the Faculty of Veterinary Medicine of Ghent University (Permit Nr. EC2010/030) and were performed in the Ghent university stables under the supervision of a veterinary doctor.

4.3.2. Salivary antigens and recombinant proteins

Saliva of *G. m. morsitans* from the ITM tsetse fly colony was collected as outflow from the salivary glands as described elsewhere (Caljon et al., 2012). Saliva of *Tabanus yao* was kindly provided by Prof. Ren Lai (Kunming Institute of Zoology, Yunnan, China), *Stomoxys calcitrans* saliva was harvested from flies received from Dr. Christopher J. Geden (United States Department of Agriculture, Gainesville, US). Recombinant Tsal1 was purified as described elsewhere from inclusion bodies of IPTG-induced Top10F' *Escherichia coli* host cells harboring a pQE60:Tsal1 plasmid (Caljon et al., 2006b). Tsal1 was resolubilized in 6M guanidinium hydrochloride, enriched by Ni-NTA affinity chromatography (Qiagen) and further purified on a Superdex 200 size exclusion column connected to an Akta Explorer (GE Healthcare) in 6M ureum 50 mM Tris pH 8.0 and 600 mM NaCl. Protein concentrations were determined using Nanodrop spectrophotometry and samples were stored in aliquots at −20°C. *E. coli* soluble extract was prepared as an additive for the porcine ELISA assay diluent as described for other porcine assays (Assana et al., 2010). The extract was made from a 1L overnight culture of Top10F' *E. coli* in Terrific broth. The bacterial pellet was resuspended in 12 ml PBS supplemented with a Complete protease inhibitor cocktail tablet (Roche), followed by 5 rounds of 1 minute sonication. The soluble fraction was harvested as the supernatant after 30 minutes centrifugation at 20.000× *g. E. coli* soluble extract was stored in aliquots at −20°C.

4.3.3. Experimental study animals and immunization

Groups of eight female outbred mice (NMRI, Charles River) were subjected to different intensities of exposure to *G. m. morsitans* bites, followed by regular blood sampling and evaluation of the antibody responses in ELISA: (*i*) once exposed to a single fly, (*ii*) once exposed to 10 flies, (*iii*) 3 times per week for 3 weeks exposed to a single fly, (*iv*) 3 times per week for 3 weeks exposed to 10 flies and (*v*) not exposed to tsetse fly bites. One mouse that underwent multiple exposures to a single fly succumbed by day 28 after initiation of exposure. Plasma was collected over a period of 390 days. After this >1 year non-exposure period, six mice that were exposed to single or multiple (10) flies were selected based on their physical appearance and behavior and were re-exposed to a single *G. m. morsitans* tsetse fly followed by weekly plasma collection for up to 42 days after re-challenge. Two mice that were previously exposed to the multiple fly bites succumbed within 4 weeks after the boosting.

A *Glossina* species cross-reactivity study was performed by exposing five mice (OF1, Charles River) per group every 3 days for 6 weeks to 10 flies of either *G. m. morsitans*, *G. p. gambiensis*, *G. pallidipes* (kindly provided by Peter Takac, Institute of Zoology, Bratislava, Slovakia) or *G. f. fuscipes* (kindly provided by the International Centre of Insect Physiology and Ecology, Mbita Point, Kenya). Immune plasma was harvested 10 days after the last exposure. Five non-exposed mice served as negative controls. A cross-reactivity study between salivary antigens of hematophagous insects was performed by intrapinna exposure of 6 OF1 mice per group at 3-weekly interval to decreasing amounts of saliva (5, 2, 1 and 1 µg) harvested from *G. morsitans*, *Stomoxys calcitrans* and *Tabanus yao*. Plasma samples were collected 10 days after the last immunization.

A total of 11 female pigs (Seghers, Belgium), hybrids of Belgian Landrace, Large White and a specific company line were used for experimental exposure to three different *G. m. morsitans* exposure regimens: (*i*) 5 pigs were weekly exposed for 7 weeks to 30 tsetse flies (high exposure), (*ii*) 5 pigs were exposed once in two weeks for 6 weeks to 3 tsetse flies (low exposure, 1 pig died at day 7) and (*iii*) 1 pig was not exposed to tsetse (negative control). The experimental pigs were 6 weeks old at the start of the experiment following a 4-days acclimatisation period in the university stables (Faculty of Veterinary Medicine, UGhent). For anaesthesia of the pigs, intramuscular injection of midazolam (0.5 mg/kg), ketamine (10 mg/kg) and morphine (0.1 mg/kg) was used. Blood was collected every week for 11 successive weeks from the jugular vein using Vacutainer EDTA tubes (BD) a few minutes after the pigs had been anesthetised and prior

to exposure to the tsetse fly bites as shown in Figure 4.1. After blood collection, the blood was centrifuged at 3000 rpm for 15 minutes and plasma stored at −20°C. Due to the unexpected death of 1 pig, no tsetse challenge and blood sampling was performed on day 7 for all animals.

Due to limited housing capacity in the university stables, only two pigs from the low exposure group could be kept for an additional 2-month period of non-exposure, followed by a re-challenge by the bites of 10 *G. m. morsitans* flies and a weekly plasma collection over a period of 6 weeks.

Figure 4.1. Exposure of anesthetised pigs to the tsetse fly bites.

A total of 13 calves of Zebu were kept in fly proof experimental house at the College of Veterinary Medicine of the Addis Ababa University. *G. pallidipes* flies were weekly obtained from Kaliti tsetse mass rearing factory. The 13 calves were divided into three groups: (*i*) a high challenge group (6 calves) with each calf exposed to 100 *G. pallidipes* every week for 6 weeks, (*ii*) low challenge group (6 calves) with each calf exposed to 10 *G. pallidipes* every two weeks for 6 weeks, and (*iii*) one naïve calf as a negative control. Serum collection was undertaken for 3 months starting at day zero, then every 3 days for the first month and every week for the last two months. Antibody responses were evaluated using indirect ELISA with the aim to optimize the test for cattle under field condition. Accordingly, cattle sera were collected from high, low and none tsetse challenge areas in Ethiopia.

4.3.4. Serological analyses

IgG responses in the exposed animals were analyzed by indirect ELISA against rTsal1 and saliva from different hematophagous insects. For this purpose, polystyrene 96 well plates (Thermo Scientific NUNC MaxiSorp Surface) were coated overnight at 4°C with 200 ng antigen (*G. morsitans*, *S. calcitrans* or *T. yao* saliva or rTsal1) per well in 0.1 M $NaHCO_3$ (pH 8.3). Plates were overcoated for 1 h with 10% fetal bovine serum (FBS) at ambient temperature. Serial half plasma dilutions starting from 1:100 in assay diluent (PBS/10%FBS) were applied for 2 h to antigen and FBS-coated wells. For the analysis of porcine plasma samples, 20% Top10F' *E. coli* soluble extract was added to the assay diluent to reduce unspecific binding to the antigenic coat. Based on the plasma dilution experiments, a 1:1600 dilution was chosen for the time course analyses. Specific immunoglobulin detection was achieved using horseradish peroxidase conjugated detection antibodies. For the detection of mouse and porcine IgGs respectively a 1:1000 diluted rabbit $F(ab)_2$ anti-mouse IgG (STAR13B, Serotec) and a rabbit anti-pig IgG conjugate (A5670, Sigma) 1:4000 diluted in PBS/10%FBS were used. Detection was with TMB substrate (3,3′,5,5′-Tetramethylbenzidine, Sigma) and reactions were stopped by the 1:3 addition of 1N H_2SO_4. Optical density (O.D.) was measured using a Multiskan Ascent plate reader (Thermo) at a 450 nm wavelength.

4.3.5. Data analysis

Antigen-specific responses were expressed as the ΔO.D. between antigen and non-antigen-coated wells. Statistical analyses were performed in SAS Version 9.3 (SAS Institute Inc., Cary, NC, USA). The effect of tsetse fly exposure intensity and boosting on anti-*G. m. morsitans* saliva and anti-rTsal1 immune responses in mice and pigs was analysed over the entire sampling period based on a mixed model with animal as random effect and challenge, time and their interaction as categorical fixed effects and F-tests were performed at the 5% significance level. Pairwise comparisons were performed using Tukey's multiple comparisons technique to adjust the significance level. Cross-reactivity of immune responses induced by different *Glossina* species and other hematophagous insects was analysed using a linear fixed effects model using the 1:100 or 1:1600 dilution with challenge as categorical fixed effect. Pairwise comparisons were performed with control using Dunnett's multiple comparisons technique to adjust the significance level. Intra-laboratory repeatability of the ELISA tests was assessed by the non-parametric Spearman correlation test. Sensitivity and specificity of the assays were assessed by receiver operating characteristic (ROC) curve analysis of the ΔO.D. values of exposed and non-exposed

animals, starting from 3 weeks after the initial exposure. The area under the ROC curve (AUC) was used as a global index of diagnostic accuracy. Kinetics of the IgG clearance in mice was assessed in five mice from the multiple exposure schemes with sufficient remaining plasma for the different timepoints and using the plasma sample with the highest antibody titer (set as 100%) to prepare a standard curve. Percent decrease in antibody titers over time was assessed by a two-phase non-linear regression allowing the estimation of the antibody half-life.

4.4. Results

4.4.1. Serological responses in mice exposed to *Glossina morsitans*

Induction of specific antibody responses was assessed by indirect ELISA in mice following different regimens of exposure to *G. m. morsitans* bites. Anti-*G. m. morsitans* saliva and anti-rTsal1 IgGs were detectable within 7 days after the initial exposure and remained persistently detectable up to 390 days (Figure 4.2). The detected IgG titers against both antigens correlated with the different intensities of tsetse fly exposure. Differences in number of flies (1 versus 10 flies) as well as differences in frequency (single versus weekly exposure over 3 weeks) were detected by ELISA. Exposure to a single tsetse fly bite was sufficient to induce slightly elevated levels of antibodies against *G. m. morsitans* saliva and rTsal1 (Figure 4.2), although the recorded differences with control plasma were not significant (p=0.9843 and p=0.6146 for the anti-rTsal1 and anti-*G. m. morsitans* saliva tests respectively). Single exposure of mice to 10 flies resulted in significantly increased reactivity against *G. m. morsitans* saliva (p=0.0160) but not against rTsal1 (p =0.8618). With both antigens, statistically significant differences were recorded considering the entire sampling period between control mice and mice subjected to the repeated exposure to 1 and 10 flies (p<0.0001). Both the anti-rTsal1 and the anti-*G. m. morsitans* saliva ELISA test were able to differentiate between these two repeated tsetse exposure schemes (p<0.0001 and p =0.0012, respectively).

Based on a standard curve generated using the plasma sample with the highest IgG titer (i.e. day 28, mouse exposed to the most intense biting regimen), antibody half-lives in five mice exposed multiple times to a single fly or 10 flies were determined by two-phase decay regressions (Figure 4.3).

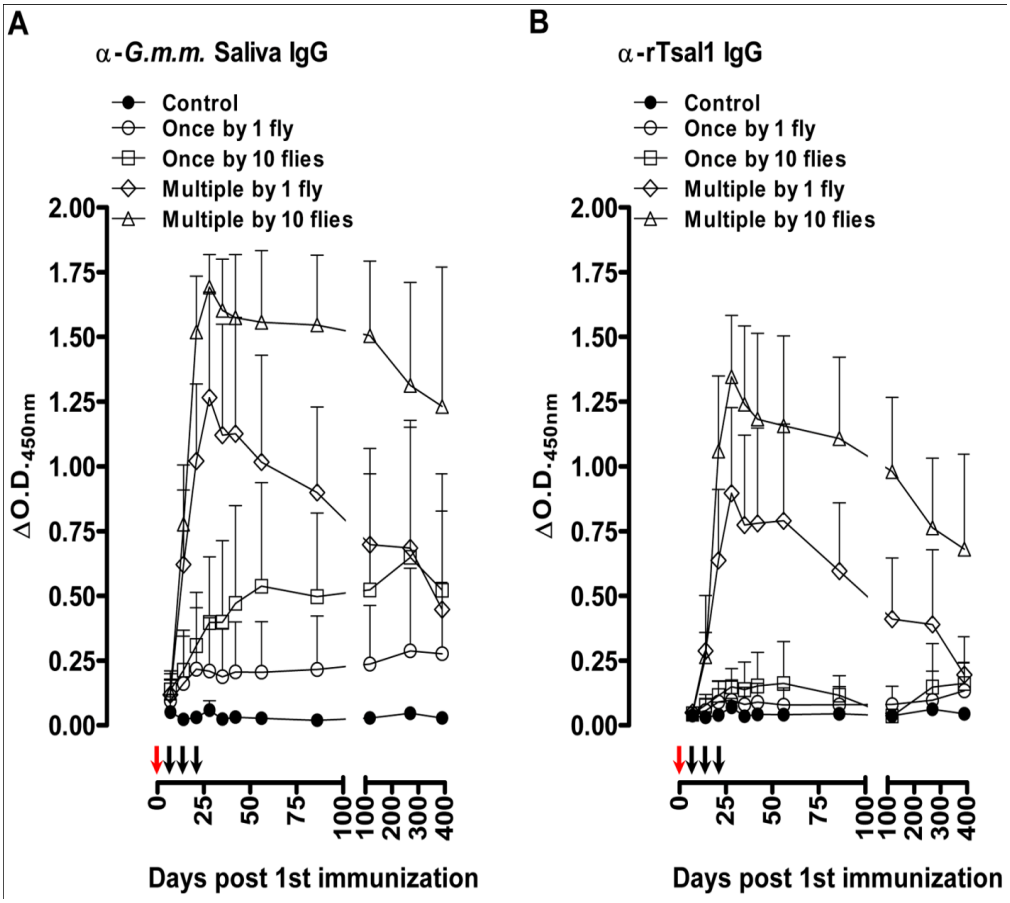

Figure 4.2. Tsetse fly induced humoral responses in mice. Long-term follow-up of (**A**) the anti-G. m. morsitans saliva IgG responses and (**B**) the anti-rTsal1 IgG responses in mice (n =8/group) exposed to 4 different tsetse fly biting intensities (1 versus 10 flies) and frequencies (single versus weekly exposure for 3 weeks). Arrows indicate the tsetse exposure regimen for single exposed (day 0) and multiple exposed mice (day 0, 7, 14, 21). Presented data are the mean $\Delta O.D._{\cdot 450\ nm}$ values and the 95% CI obtained with a 1:1600 dilution of the individual plasma.

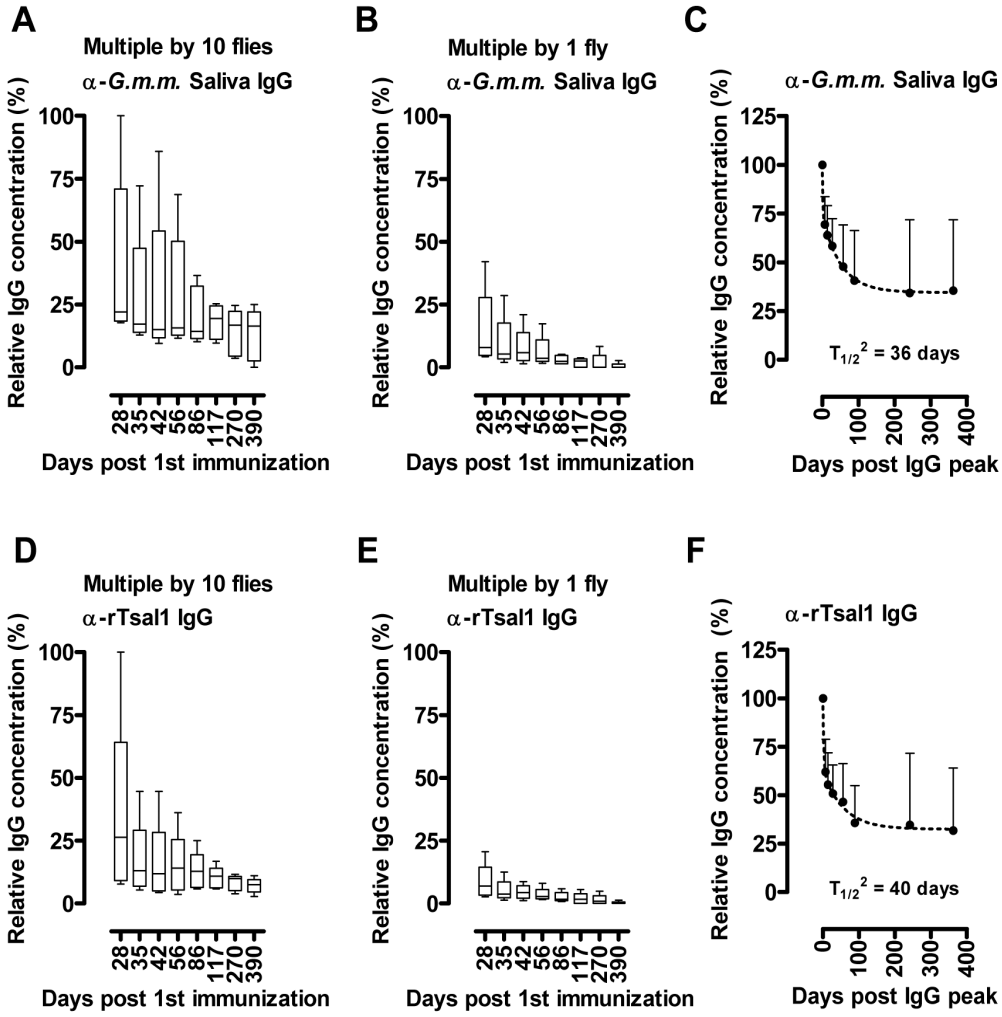

Figure 4.3. Antibody kinetics in tsetse exposed mice. Evolution over time in the absence of tsetse fly challenge (from day 28 onwards) of the anti-G. m. morsitans saliva IgG concentrations (**A, B, C**) and the anti-rTsal1 IgG concentrations (**D, E, F**) in mice (n=5/group) exposed multiple times to a single fly (**B, E**) or 10 flies (**A, D**). Presented data are the IgG concentrations relative to the highest responder plasma and expressed as percent based on a standard dilution series. Box plots represent the means, with minimum and maximum and the 75-25% percentiles. The average antibody half-life ($T_{1/2}^2$, **C, F**) over the 2 exposure groups (n =10) was determined by a two-phase non-linear decay regression (dotted line) of the declining antibody concentrations relative to the concentration recorded after cessation of the tsetse exposure (day 28 set as starting concentration of 100%). Scatter plots indicate the mean concentrations with the 95% CI.

The average half-lives ($T_{1/2}^2$) of the anti-saliva and anti-rTsal IgGs in a second phase of decay after a first phase immediately after cessation of tsetse exposure were respectively 36 and 40 days (Figure 4.3). There was a significant individual variation within the different exposure groups with up to a 10-fold difference between the strongest and weakest responder. On average, a 3 to 4-fold difference in peak IgG titer was recorded between mice exposed to a low (multiple bites by a single fly) and high exposure regimen (multiple bites by 10 flies).

The antibodies appeared relatively persistent over an evaluation period of more than 1 year. Following a long period of non-exposure, the bite of a single tsetse fly was sufficient to boost the anti-saliva and anti-rTsal1 IgG titers. This boosting appeared independent of the previous exposure intensity (Figure 4.4) as statistical analysis over the 6-week sampling period revealed the inability of the saliva and rTsal1-based tests to differentiate mice subjected to a low or high initial exposure regimen (p=0.4141 and p =0.9609 respectively). Antibody titers against both antigens reached a plateau within 7 days after re-challenge, while in naive animals the responses were slightly lower (p=0.0833 and p= 0.1925 for the anti-saliva and anti-rTsal1 IgG levels respectively) and only reached peak titers within 4 weeks after initial exposure (Figure 4.4).

A strong correlation between anti-saliva and anti-rTsal1 ELISA results was recorded with a Spearman correlation coefficient r of 0.92. Intra-laboratory repeatability of the anti-saliva and anti-rTsal1 detection assays was excellent (Spearman r =0.98). A comparison of the diagnostic value of the anti-saliva and rTsal1 indirect ELISA was conducted using ROC curve analysis. Comparison of the area under the curve (AUC), based on the mouse plasma from the experiment presented in Figure 4.2 and collected at least 3 weeks after the initial tsetse fly exposure, indicated that the anti-tsetse saliva ELISA has a better test performance than the rTsal1-based assay (AUC 0.94 versus 0.82, Figure 4.5).

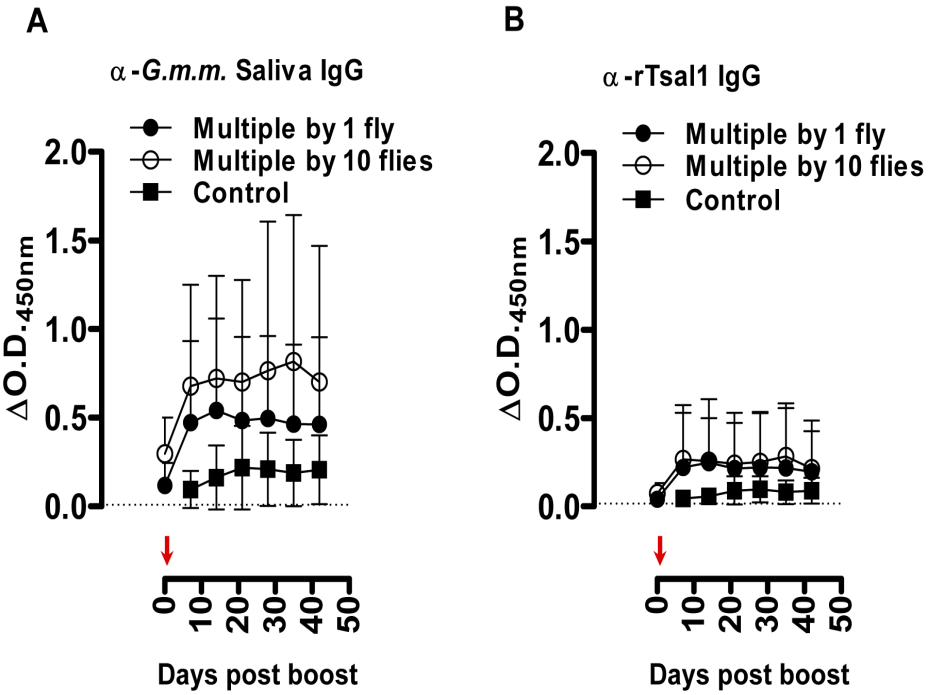

Figure 4.4. Effect of boosting in mice after a long period of non-exposure. Evolution of the anti-G. m. morsitans saliva IgG responses (**A**) and the anti-rTsal1 IgG repsonses (**B)** was evaluated in mice (n =6/group) originally exposed to the bites of a single or 10 flies and re-exposed to a single tsetse fly bite after a 1-year period of non-exposure (indicated by an arrow). Controls are the naive mice exposed to a single bite as depicted in Figure 4.2. The original control animals (n=3) were not exposed and served as negative controls (dotted line). Presented data are mean $\Delta O.D._{450\ nm}$ values with the 95% CI obtained with a 1:1600 dilution of the individual plasma.

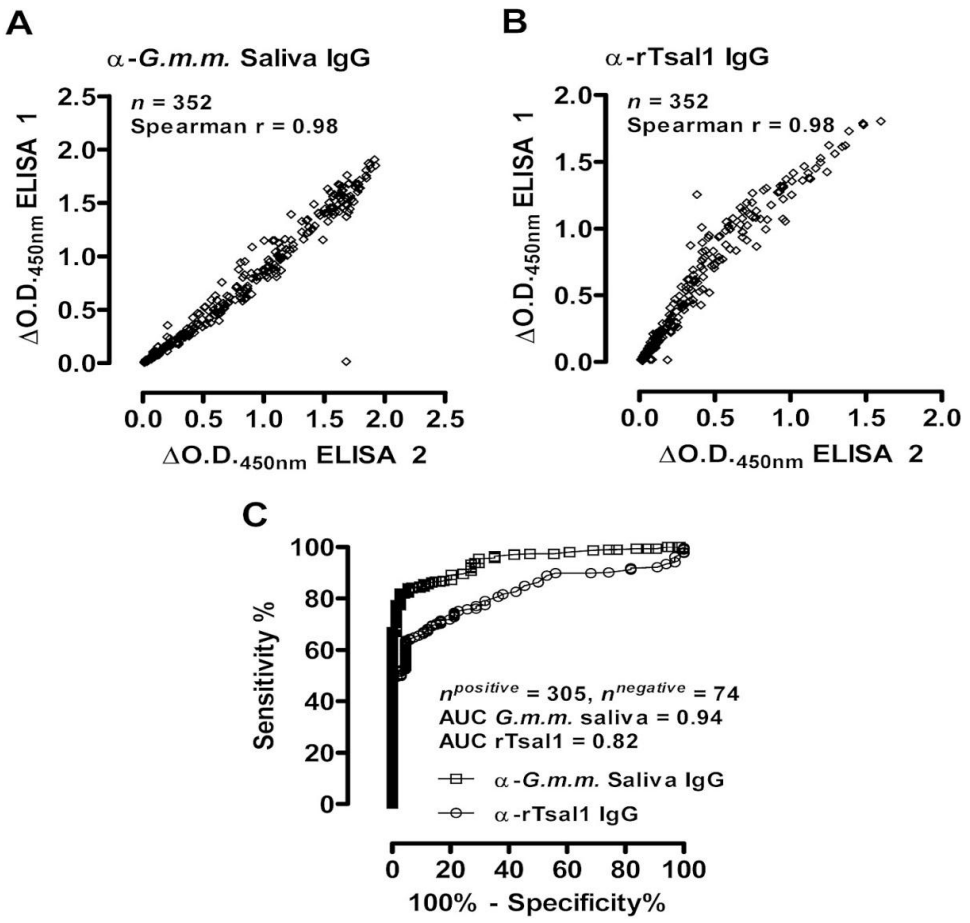

Figure 4.5. Repeatability and specificity/sensitivity analysis of the antibody detection test in mice. Scatter plot analysis of the anti-G. m. morsitans saliva IgG responses (**A**) and the anti-rTsal1 IgG repsonses (**B**) ($\Delta O.D._{450\ nm}$) in two separate tests performed on a panel of 1:1600 diluted mouse plasma samples (n = 352). Test repeatability was analyzed by the non-parametric Spearman correlation test. Sensitivity and specificity of the two assays were assessed by receiver operating characteristic (ROC) curve analysis of the ΔO.D. values of exposed and non-exposed mice (**C**). The area under the ROC curve (AUC) is reported as a measure for the test performance.

4.4.2. Serological responses of mice exposed to various *Glossina* species

Mice were repeatedly exposed to the bites of various *Glossina* species (*G. m. morsitans*, *G. p. gambiensis*, *G. pallidipes* and *G. f. fuscipes*). It was noted that the feeding performance of *G.*

fuscipes on mice was inferior to those of the other species, resulting in a high mortality of these flies when maintained on mice. *G. m. morsitans* saliva and rTsal1 were next used as antigens in indirect ELISA to detect the elicited antibodies. A marked cross-reactivity of the anti-saliva IgGs elicited by three *Glossina* species (*G. m. morsitans, G. p. gambiensis* and *G. pallidipes*, $p<0.0001$ at the 1:100 plasma dilution) and a relatively weaker cross-reactivity of *G. f. fuscipes* ($p=0.0053$) was detected with *G. m. morsitans* saliva as antigen (Figure 4.6A&B). Similarly, antibodies induced by exposure to *G. m. morsitans, G. p. gambiensis* and *G. pallidipes* significantly reacted with rTsal1 (respectively $p<0.0001$, $p=0.0157$ and $p=0.0002$), but no cross-reaction with rTsal1 was detected in mice exposed to *G. f. fuscipes* ($p=0.9883$). This indicated that a number of antigens are sufficiently conserved in the saliva of several *Glossina* species to allow multi-species detection of exposure using *G. m. morsitans* saliva. The rTsal1 also enabled detection of exposure of mice to various tested tsetse fly species, except for *G. fuscipes* (*Palpalis* subgenus).

4.4.3. Serological responses of mice exposed to various hematophagous insect species

Mice were experimentally immunized by 4 intradermal injections with the saliva of stable flies (*Stomoxys calcitrans*) or horse flies (*Tabanus yao*) and compared with control mice and mice that were immunized with tsetse fly saliva following the same immunization protocol. Immunization with *Tabanus* saliva did not result in IgGs that reacted with rTsal1 and total *G. morsitansmorsitans* saliva as coating antigens ($p=0.7882$ and $p=0.7637$ at the 1:100 plasma dilution (Figure 4.6D&E). However, a slight increase that did not reach statistical significance was observed in responsiveness of the *Stomoxys* exposed plasma in the rTsal1 and saliva-based assays ($p=0.0837$ and $p=0.0639$ respectively). At a standard 1:1600 dilution, no cross-reaction of the *Stomoxys* exposed plasma with rTsal1 and saliva was detected ($p=0.9920$ and $p=0.9915$ respectively). Analysis of the published salivary transcriptome of *Stomoxys calcitrans* suggested the presence of a Tsal1 homologue from which a truncated sequence was published (GenBank Accession N°: ACN69159, (Wang et al., 2009). Within this region, only 36% identity in amino acid sequence was found, which could explain the slightly elevated anti-rTsal1 reactivity of *Stomoxys* exposed mice.

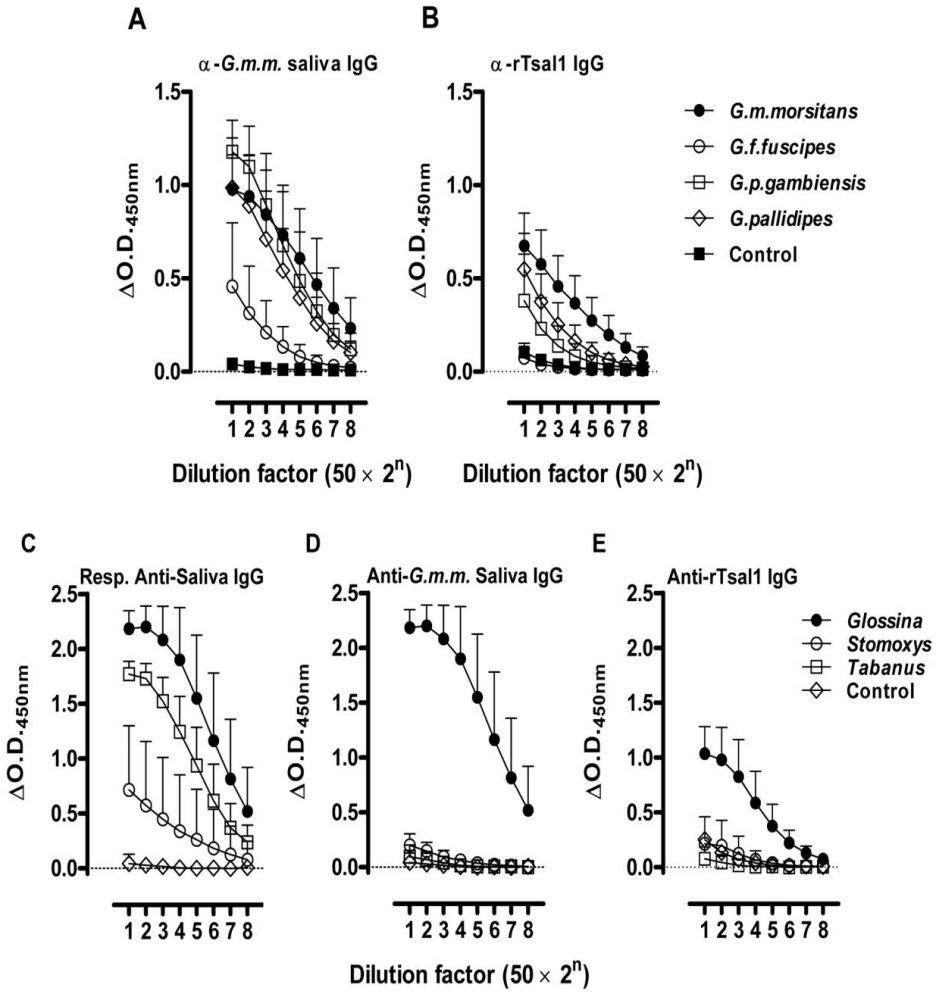

Figure 4.6. Detection of humoral responses induced by various hematophagous insects. Serially diluted plasma of control mice and mice repeatedly exposed to the bites of G. morsitansmorsitans morsitans, G. fuscipes fuscipes, G. palpalis gambiensis and G. pallidipes (n=5/group) were tested in the G. m. morsitans saliva-based (**A**) and rTsal1-based IgG detection ELISA (**B**). Serially diluted plasma of control mice and mice experimentally immunized with harvested G. m. morsitans, Stomoxys calcitrans and Tabanus yao saliva (n=6/group) were analysed for responses against the respective saliva extracts (**C**) and were tested in the G. m. morsitans saliva-based (**D**) and rTsal1-based IgG detection ELISA (**E**). Presented data are the mean $\Delta O.D._{450\ nm}$ values with the 95% CI.

4.4.4. Serological responses in tsetse fly exposed pigs

Pigs were experimentally exposed to a low or a high tsetse fly bite regimen, followed by assessment of the antibody production. As compared to the mouse indirect ELISA, we have modified the porcine assay by including *E. coli* soluble extract in the sample diluent as described elsewhere (Assana et al., 2010) to reduce the background that was observed particularly onto the rTsal1 antigen which was produced in a bacterial expression system. Under the used assay conditions, anti-rTsal1 and anti-saliva IgGs were detected from day 21 after the first tsetse exposure onwards (Figure 4.7). The specific IgG titers in pigs correlated with the intensity of tsetse exposure. Anti-rTsal1 and anti-tsetse saliva IgGs were elevated in tsetse exposed groups as compared to the non-exposed control pig and pre-immune plasma, with significant differences for the high exposure group (p=0.0216 and p=0.0030 respectively). The repeated exposure of pigs to 30 flies resulted in higher anti-rTsal1 and anti-tsetse saliva IgG titers as compared to the pigs exposed to a low tsetse challenge by 3 flies (p=0.0014 and p=0.0024 respectively). However, exposure to the low exposure scheme did not result in significantly elevated responses in both the rTsal1 and saliva-based ELISA (p=0.8844 and p=0.4261 respectively, Figure 4.7).

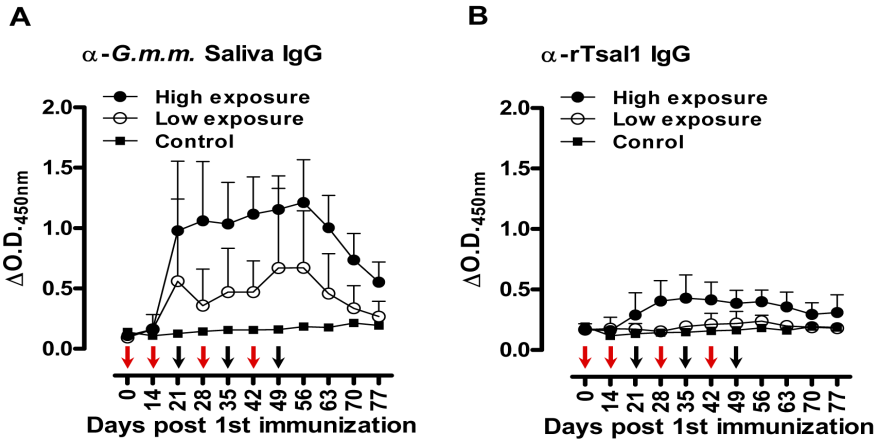

Figure 4.7. Tsetse fly induced humoral responses in pigs. Follow-up of the anti-G. m. morsitans saliva IgG responses (**A**) and the anti-rTsal1 IgG responses (**B**) in pigs exposed to 2 different tsetse fly biting intensities (two-weekly exposure for 6 weeks to 3 flies (low exposure group, n=4) or weekly exposure for 7 weeks to 30 flies (high exposure group, n=5)). One non-exposed animal was included as a negative control. Arrows indicate the tsetse exposure regimen for the low exposure (0, 14, 28 & 42 arrows) and high exposure group (day 0, 14, 21, 28, 35, 42 & 49 arrows). Presented data are the mean $\Delta O.D._{450\ nm}$ values and the 95% CI obtained with a 1:1600 dilution of the individual plasma.

Boosting of 2 pigs from the low exposure group after a 2-month non-exposure period by the bites of 10 flies resulted in elevated anti-saliva IgG titers but was only weakly detectable using the rTsal1-based ELISA and with higher individual variation (Figure 4.8).

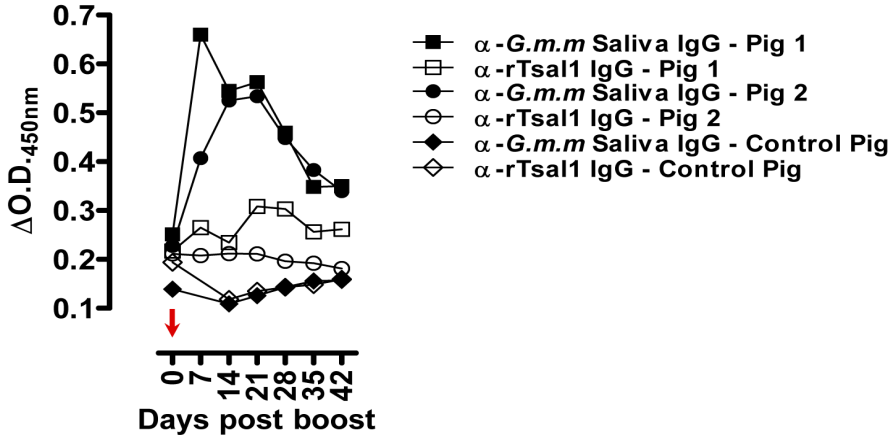

Figure 4.8. Effect of boosting in pigs after a period of non-exposure. Evolution of the anti-G. m. morsitans saliva IgG responses and the anti-rTsal1 IgG repsonses was evaluated in pigs (n =2) originally exposed two-weekly for 6 weeks to the bites of 3 flies (=low exposure group) and re-exposed to the bites of 10 flies (indicated by an arrow) after a 2-month period of non-exposure. One non-exposed animal sampled during the priming experiment (Figure 4.7) served as a negative control. Presented data are mean $\Delta O.D._{450\ nm}$ values with the 95% CI obtained with a 1:1600 dilution of the individual plasma.

In general, a good correlation between anti-saliva and anti-rTsal1 ELISA results, obtained for the samples from the tsetse fly exposure experiment presented in Figure 4.7, was recorded with a Spearman correlation coefficient *r* of 0.79. Intra-laboratory repeatability of the anti-saliva and anti-rTsal1 detection assays was adequate (Spearman *r* =0.94). Comparison of the area under the curve (AUC) for the anti-tsetse saliva and anti-rTsal1 ELISA ROC curve indicated that the assay based on total *G. morsitansmorsitans* saliva has a better average performance than the anti-rTsal1 IgG detection test (AUC 0.96 versus 0.83, Figure 4.9).

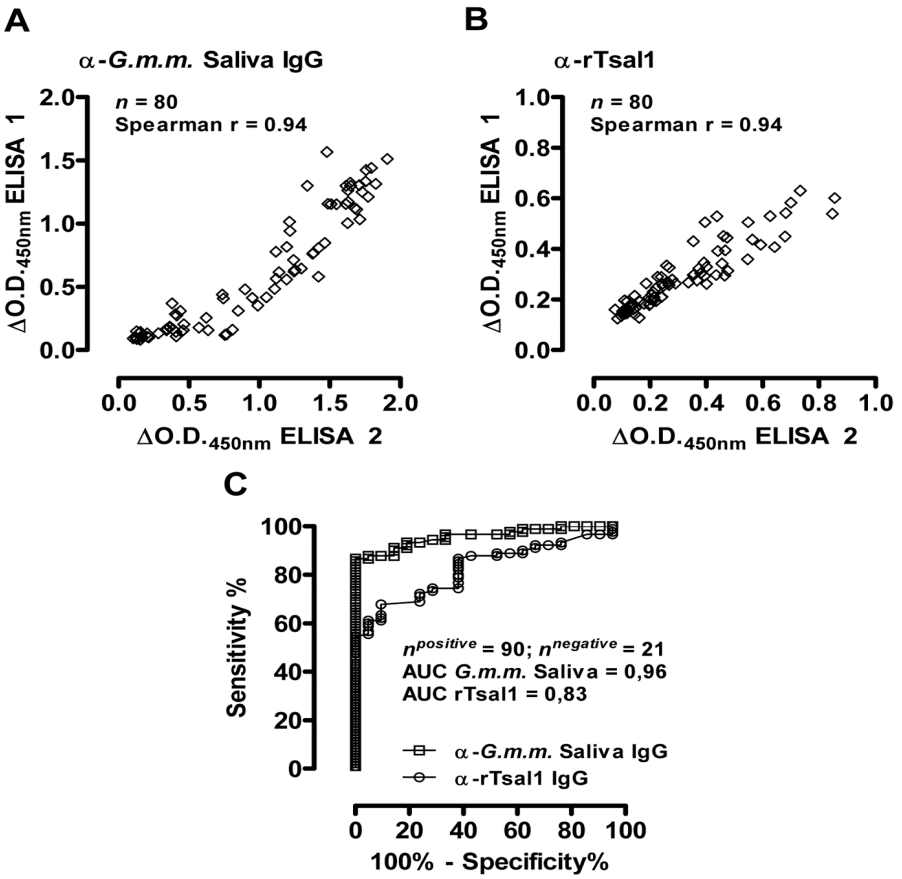

Figure 4.9. Repeatability and specificity/sensitivity analysis of the antibody detection test in pigs. Scatter plot analysis of the anti-G. m. morsitans saliva IgG responses (**A**) and the anti-rTsal1 IgG repsonses (**B**) ($\Delta O.D._{450\ nm}$) in two separate tests performed on a panel of 1:1600 diluted porcine plasma samples (n = 80). Test repeatability was analyzed by the non-parametric Spearman correlation test. Sensitivity and specificity of the two assays were assessed by receiver operating characteristic (ROC) curve analysis of the $\Delta O.D.$ values of exposed and non-exposed mice (**C**). The area under the ROC curve (AUC) is reported as a measure for the test performance.

4.4.5. Serological responses in tsetse fly exposed calves

The sera of cattle in both total saliva and rTsal1 ELISA test gave high background binding thus hiding specific binding, for still unknown reason. Thus, the test could not be optimized for cattle which is the most important livestock of Africa. The low specificity could be due to cross-reactivity with other biting insects (*Tabanus* and *stomoxys* sp.).

4.5. Discussion

Assessment of exposure of populations at risk to the bites of tsetse fly vectors could be an important step towards improved control of both HAT and AAT. Given that novel innovative vector control strategies are being developed (e.g. miniaturized insecticide-treated targets (Lindh et al., 2009), the release of sterile male insects (Vreysen et al., 2000; Sow et al., 2012) and deployed on increasingly large scales on the African continent, monitoring the impact of these new as well as conventional interventions on actual exposure to tsetse fly bites is a logical follow-up. The tsetse fly species implicated in the transmission of trypanosomes are not uniform throughout the African continent as more than 30 tsetse species and subspecies exist that play differential roles in parasite transmission, have preferences for specific biotopes and display specific host feeding preferences. A number of species are strongly implicated in HAT transmission, such as flies of the *morsitans* subgenus (e.g. used in this study: *G. morsitans morsitans*, *G. pallidipes*) and flies of the Palpalis group (e.g. used in this study: *G. palpalis gambiensis*, *G. fuscipes fuscipes*). In case of AAT, a large panel of tsetse species and other sympatric hematophagous arthropods are respectively involved in biological and mechanical transmission. Especially stable flies (*Stomoxys* sp.) and horse flies (*Tabanus* sp.) play a role in mechanical transmission of animal trypanosomes such as *T.vivax*. However, these insects are not able to biologically transmit trypanosomes as the parasite cannot complete its life cycle in these insects.

Research on a number of hematophagous arthropod vectors has resulted in the concept of exploiting salivary components as specific biomarkers of exposure (Schwartz et al., 1990; Volf et al., 1993; Rohousova et al., 2005; Caljon et al., 2006b; Remoue et al., 2006; Schwarz et al., 2009; Drame et al., 2010a). An advantage of this approach is that relatively simple serological tests could provide information on actual exposure to the bites of disease vectors and provide a risk indicator of contracting a vector-transmitted disease without the need of strenuous entomological surveys. Whole salivary gland extracts of sand flies (Rohousova et al., 2005, Vinhas et al., 2007; Gidwani et al., 2011), triatomine bugs (Volf et al., 1993; Schwarz et al., 2009) and various mosquito species (Orlandi-Pradines et al., 2007; Drame et al., 2010b; Doucoure et al., 2012) can be used to assess biting exposure. It has been suggested that this type of serological approach would enable the detection of very low levels of exposure that could remain undetected by entomological trappings (Poinsignon et al., 2009). Also differences in exposure level due to vector control interventions (e.g. insecticide treated bednets) could be elucidated on the basis of

salivary proteins as immunological probes (Drame et al., 2010a; Gidwani et al., 2011; Schwarz et al., 2011; Sagna et al., 2013). While total salivary extracts can be used in various ELISA and immunoblot formats, some studies have moved towards the use of recombinant proteins or peptides which could lead to the development of more standardized immune assays (Sanders et al., 1998; Poinsignon et al., 2008b; Drame et al., 2010a; Souza et al., 2010; Teixeira et al., 2010; King et al., 2011; Ali et al., 2012; Ndille et al., 2012; Vlkova et al., 2012). Several strategies could be envisaged either aiming at species-specific or pan-species exposure detection.

Studies using various tsetse fly species have shown that salivary components are immunogenic in mice, rabbits, cattle and humans (Ellis et al., 1986; Caljon et al., 2006b; Poinsignon et al., 2007 & 2008a; Dama et al., 2013a; Somda et al., 2013). Immunoblotting of salivary proteins separated on 1D or 2D protein gels have highlighted immunogenic proteins corresponding to several protein families including endonuclease (Tsal), adenosine deaminase (TSGF), 5′nucleotidase (5′Nuc) and Antigen 5 (Ag5) related proteins (Dama et al., 2013a). In addition, the immunogenic nature of *G. m. morsitans sgp1*, *sgp2* and *sgp3* resulted in their identification on the basis of immune screening of a phage cDNA expression library (Van Den Abbeele et al., 2007). We have previously observed by Western blot analysis that human plasma samples collected in Tororo of Uganda (MacLean et al., 2006) where *G.fuscipes fuscipes* is the predominant tsetse fly, commonly recognized the 43–45 kDa Tsal proteins in *G. m. morsitans* saliva (Caljon et al., 2006b). This suggested that *G. m. morsitans* Tsal-based immune screening of these Ugandan samples cross-detected exposure to *G. fuscipes fuscipes*, responsible for *Trypanosoma brucei rhodesiense* transmission in that area (Aksoy et al., 2013). Plasma samples from tsetse fly exposed individuals in Guinean HAT foci also displayed strong reactivity against the highly abundant Tsal proteins in *G. palpalis gambiensis* saliva in immunoblots (Dama et al., 2013a). As the Tsal protein band is commonly recognized in samples from *Glossina* -exposed humans (Caljon et al., 2006b; Dama et al., 2013a), Tsal proteins could have the potential of a pan-*Glossina* species exposure marker. However, reactivity with Tsal1 and Tsal2 was also observed with a pool of human control plasma from Bobo-Dioulasso (Burkina Faso) (Dama et al., 2013a). Based on our current study we anticipate that the inclusion criteria for these urban unexposed plasma donors (not having traveled outside of the city for at least three months, (Dama et al., 2013b) might have been insufficiently stringent to exclude circulating anti-Tsal antibodies. Indeed, tsetse flies are present at sites outside the city and given the high immunogenicity and anticipated long antibody persistence, this could explain the documented positive reactions in the immunoblots.

Production of a recombinant version of the Tsal1 protein allowed evaluating its effectiveness for detecting tsetse fly exposure in an antibody-detection ELISA. By experimentally exposing mice and pigs to different tsetse fly biting regimens, the high immunogenicity of total *G. m. morsitans* saliva and Tsal1 as a major constituent was confirmed. In mice, a single bite was proven sufficient to induce a detectable immune response in naive animals and to boost antibody levels in previously exposed animals. Kinetics of the antibody clearance was assessed in mice over a >1 year period, which revealed a long persistence with an average half-life of 36–40 days. In pigs, the apparent antibody clearance rate was faster and although the time of sampling and the number of experimental animals was limited, the anti-saliva IgG half-life could be estimated to be 15 days (data not shown). This clearance rate was consistent with the fast decline of anti-*G. m. submorsitans* saliva IgG levels observed in cattle within 10 weeks after cessation of tsetse exposure (Somda et al., 2013). Both in mice and pigs, boosting of prior induced anti-saliva immunity was observed within 7 days by the anti-*G. m. morsitans* saliva and anti-rTsal1 ELISA. However, with the used sample sizes and experimental conditions, both the rTsal1 and saliva-based ELISA were unable to statistically differentiate the control pig from pigs exposed to a low tsetse fly challenge. Interestingly, the boosting of immunity in mice seemed independent of the previous exposure intensity. As such, surveys based on rTsal1 or total *G. m. morsitans* saliva in tsetse fly control intervention zones would give information on the actual tsetse exposure intensity rather than history and could provide a tool to monitor re-invasion. Consistent with this, serological results for cattle in South-West Burkina Faso based on *G. palpalis gambiensis* whole saliva extract related to the seasonality which directly impacts the intensity of host/vector contact (Somda et al., 2013). Also humans living in HAT endemic areas display significant differences in anti-*G. p. gambiensis* saliva IgGs depending on the study site (Dama et al., 2013a). Similar results were obtained with a peptide derived from the adenosine deaminase-related protein TSGF1, although obtained ELISA signals seemed very low (Dama et al., 2013b). In both the cattle and human ELISA tests, large inter-individual differences were observed (Caljon et al., 2006b; Dama et al., 2013a & b; Somda et al., 2013). It should be noted that in our study the individual variation in antibody titers in the different exposure groups also varied significantly. In controlled experimental conditions with outbred mice, we recorded up to 10-fold differences in anti-saliva and anti-rTsal1 antibody concentrations between the strongest and weakest responders. Consequently, using the tsetse salivary antigens as immunological probes has the limitation of inherent variability due to individual differences in immunological responsiveness. Nevertheless, we observed a clear overall difference in anti-rTsal1 and anti-saliva IgG levels

between the different exposure groups of mice and pigs, with an average 3–4 fold difference in specific antibody concentration between a low exposure (repeated exposure to 1 fly for mice or to 3 flies for pigs) and a high exposure regimen (repeated exposure to 10 flies for mice or to 30 flies for pigs). This is in line with observations made for cattle that were experimentally exposed to different biting intensities (Somda et al., 2013). Beside population-level studies, individual serological follow-up of selected animals or sentinel animals using the recombinant Tsal1 or the total *G. morsitans* saliva could provide a measure for tsetse prevalence, provide evidence for the impact of an intervention strategy or could be a sensitive tool to detect re-invasion of previously cleared areas or the efficacy of a barrier protecting a cleared area. Here, rTsal1 and total *G. morsitans* saliva seem to provide an indication of exposure to a broad range of *Glossina* species. Only for *G. f. fuscipes*, detection of induced IgGs is hampered which could relate to the genetic distance of this species as member of the Palpalis group (although *G. palpalis gambiensis* was efficiently cross-detected). Nevertheless, serological responses against rTsal1 were detected in humans from Uganda principally exposed to *G. fuscipes*, which suggests that sensitivity and specificity parameters related to a single antigen may vary considerably depending on the host species. It also remains to be evaluated whether the intensity and persistence of the anti-Tsal1 IgG responses would not result in a saturation in populations persistently exposed to tsetse bites. This could possibly limit application of the rTsal1-based assay to examining subjects in tsetse-free areas that are at risk of invasion or to monitoring sentinel animals in order to evaluate tsetse presence in endemic regions.

Exposure to the saliva of other hematophagous insects (*Tabanus* and *Stomoxys* sp.) that may be abundant in areas where tsetse flies are present, revealed that *G. m. morsitans* saliva did not yield false positive signals. Observations of *Stomoxys* and *Tabanus* exposed cattle revealed that also *G. m. submorsitans* saliva shares this feature of specificity, while *Glossina tachinoides* and *G. palpalis gambiensis* saliva yielded unspecific reactions with *Tabanus* exposed plasma (Somda et al., 2013). When using rTsal1 as an antigen, a weak unspecific signal was observed with the 1:100 to 1:800 diluted plasma of mice exposed to *Stomoxys calcitrans* saliva which could be due to the presence of a homologue (GenBank Accession N°: ACN69159, Wang et al., 2009) with relatively limited degree of identity to Tsal1. Given the strong immunogenicity of the Tsal proteins and based on our ELISA analyses with the experimentally exposed pigs and mice we propose to use a 1:1600 plasma dilution in favor of an increased specificity and an overall reduced risk of detecting cross-reactive antibodies. For the porcine ELISA, *E. coli* soluble extract was added to the sample diluent to increase the specificity similar to what was proposed for other

pig serological tests (Assana et al., 2010). Under these stringent experimental conditions, the recombinant Tsal1 was able to detect the high levels of exposure to tsetse fly bites and with a good correlation with the data obtained for total *G. m. morsitans* saliva as an antigen.

Collectively and combined with our previous analyses using a large panel of East-African human plasma (Caljon et al., 2006b), this study indicates that a recombinant version of the *G. m. morsitans* Tsal1 fulfills the criteria of a candidate exposure biomarker for a broad range of tsetse fly species. We believe that the high sensitivity of the rTsal antigen and the broad species recognition could be an added value to the immunoassays that are tested in the framework of other studies based on tsetse salivary peptides. Although total saliva displayed higher performance in this study, the use of whole saliva as antigen could present several limitations in the long-term such as problems of mass production, reproducibility and specificity. To overcome this problem, recombinant Tsal ELISA assay can be standardized, feasible for large-scale production and used as an immunoassay allowing large scale monitoring of host exposure to tsetse flies.

4.6. Conclusion

Salivary proteins of hematophagous disease vectors represent potential biomarkers of exposure and could be used in serological assays that are complementary to entomological surveys. This study illustrates that a recombinant version of the highly immunogenic Tsal1 protein of the *Savannah* tsetse fly (*G. m. morsitans*) is a sensitive immunological probe to detect contact with tsetse flies. Experimental exposure of mice and pigs to different regimens of tsetse fly bites combined with serological testing revealed that rTsal1 is a sensitive indicator that can differentiate the various degrees of exposure of animals. Tsetse-induced antibodies persisted relatively long, and an efficient boosting of immunity was observed upon re-exposure. Recombinant Tsal1 is a promising candidate to detect contact with various tsetse species, which would enable screening of populations or herds for exposure to tsetse flies in various areas on the African continent. This exposure indicator could be a valuable tool to monitor the impact of vector control programs and to detect re-invasion of cleared areas by tsetse flies.

4.7. Acknowledgments

We acknowledge Peter Van Den Bossche for his involvement in the initiation of the project and who tragically died in a car accident on 11/11/2010. We wish to acknowledge Pélagie Atrou, Serge Mpouam and Kéita Moulaye who were involved as master students in parts of this project.

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Chapter 5:

A systematic review of trypanosome prevalence in tsetse flies

Adapted from

Duguma, R., Agga, G.E., Gebregzabher, W., Bekana, M., Van Leeuwen, T., Delespaux, V. and Duchateau, L., 2016. A systematic review of trypanosome prevalence in tsetse flies. *Parasites & Vectors*, (submitted).

5.1. Abstract

The optimisation of trypanosomosis control programs warrants a good knowledge of the main vector of animal and human trypanosomes in sub-Saharan Africa, the tsetse fly. An important aspect of the tsetse fly population is its trypanosome infection prevalence, as it determines the intensity of the transmission of the parasite by the vector. We therefore conducted a systematic review of published studies documenting trypanosome infection prevalence from field surveys or from laboratory experiments under controlled conditions. The microscopic examination of dissected flies (dissection method) remains the most used method to detect trypanosomes and thus constituted the main focus of this analysis. Meta-regression was performed to identify risk factors for high trypanosome prevalence in the vectors and a random effects meta-analysis was used to report the sensitivity of molecular and serological tests using the dissection method as gold standard. The overall pooled prevalence was 10.3% (95% confidence interval [CI] = 8.1%, 12.4%) and 31.0% (95% CI = 20.0%, 42.0%) for the field survey and laboratory experiment data respectively. The country and the year of publication were found to be significantly associated with the prevalence of trypanosome infection in tsetse flies. The alternative diagnostic tools applied to dissection positive samples were characterised by low sensitivity, and no information on the specificity was available at all. Improving the sensitivity and determining the specificity of these alternative diagnostic tools should be a priority and will allow to estimate the prevalence of trypanosome infection in tsetse flies in high-throughput.

Keywords: Meta-regression, systematic review, *Glossina* , trypanosome infection prevalence, diagnostic methods

5.2. Introduction

Glossina species (commonly known as tsetse flies) are the major vectors of several *Trypanosoma* species, the causative agents of animal and human African trypanosomosis, also called Nagana and sleeping sickness, respectively (Murray and Gray, 1984; Bhalla, 2002; Brun et al., 2010). Once established in the tsetse fly, trypanosomes undergo a developmental cycle within the tsetse fly with varying complexity depending on the species (Vickerman et al., 1988). The infected tsetse fly then transmits the parasite to diverse host species during its blood meal. Tsetse flies infest an area of about 10 million km^2 comprising 38 sub-Saharan African countries (Kristjanson et al., 1999). The disease constitutes a major veterinary and medical burden affecting the life of millions of people. Within affected regions, the density of the vector and the prevalence of trypanosome infections in the host is attributed to complex interactions between and among humans, domestic livestock, wildlife, tsetse flies, trypanosomes and various economic and ecological factors (Steverding, 2008; Duguma et al., 2015; Mweempwa et al., 2015).

The prevalence of trypanosome infections in the tsetse flies is often a neglected parameter probably due to the intensive labour required for its evaluation. Integrating this parameter in a monitoring program allows however a more precise evaluation of the risk of being infected in a particular region.

Dissection of flies remains the most common technique for detecting the presence of trypanosomes. Although molecular and serological techniques are assumed to detect far higher levels of genetic diversity with a higher sensitivity (Adams et al., 2010), the performance of such tests has been reported to be unsatisfactory (Solano et al., 1995; Woolhouse et al., 1996; Morlais et al., 1998; Lehane et al., 2000; Malele et al., 2003). For instance, PCR failed to detect trypanosomes in dissection positive flies or vice versa, and tsetse fly samples negative by PCR were positive by fluorescent fragment length barcoding tests even allowing the discovery of new genotypes (Hamilton et al., 2008; Adams et al., 2010).

The aim of this systematic review was to (i) synthesize the limited information on the trypanosome prevalence in tsetse flies, (ii) identify risk factors associated with high prevalence of trypanosome infections in tsetse flies and (iii) assess the sensitivity of various diagnostic methods for the detection of trypanosomes in the tsetse flies using the dissection method as gold standard.

5.3. Methods

5.3.1. Search strategy and inclusion of studies

Publications were screened in the Web of Science, PubMed and Google Scholar databases. The last search was done on July the 20th 2015. The following Boolean parameter combinations and Medical Subject Headings terms were used: "Trypanosomes" and "infection rate" and "tsetse fly or *Glossina*". The retrieved articles were then first screened by title and abstract by two independent readers. Any discrepancies were discussed until consensus. Selected articles were retained for further full text analysis. The inclusion criteria for further data extraction and meta-analysis were the presence of the following data: *(i)* tsetse species, *(ii)* study type (laboratory or field), *(iii)* location (country) of study, *(iv)* trypanosome detection method, *(v)* type of tsetse sample examined, *(vi)* number and type of fly samples, and *(vii)* number of samples positive for trypanosomes. A flow chart describing the number of articles retrieved, screened and included or rejected is presented in Figure 5.1.

5.3.2. Meta-analysis

All meta-analyses were performed with STATA Version 12 (StataCorp LP, College Station, Texas). The core analysis focused on the prevalence of trypanosome infections in the tsetse flies as assessed by the dissection technique. Analyses are done separately for data obtained from field surveys and laboratory experiments. In laboratory experiments, blood meals and external conditions are standardized and all feeding flies ingest parasites. In the field surveys, the prevalence of trypanosomes in the host and the parasitemia will be the determining factors explaining the prevalence in flies. First, pooled estimates were calculated based on the random effects model (DerSimonian and Laird, 1986; Kontopantelis and Reeves, 2010) with study as random effect. Results were presented by forest plots. Next, potential risk factors that could be associated with the prevalence of infection were considered using logistic meta-regression analysis (Harbord and Higgins, 2008; Thompson, and Higgins, 2002) with country, tsetse species and tsetse organ as categorical and year of publication as continuous fixed effects factors. Odds ratios with their 95% confidence interval (CI) were used as summary statistic and testing was done at the 5% significance level.

A second part of the meta-analysis assessed the sensitivity of the alternative diagnostic tests using the dissection method as a gold standard. The sensitivity was evaluated using the random effects model.

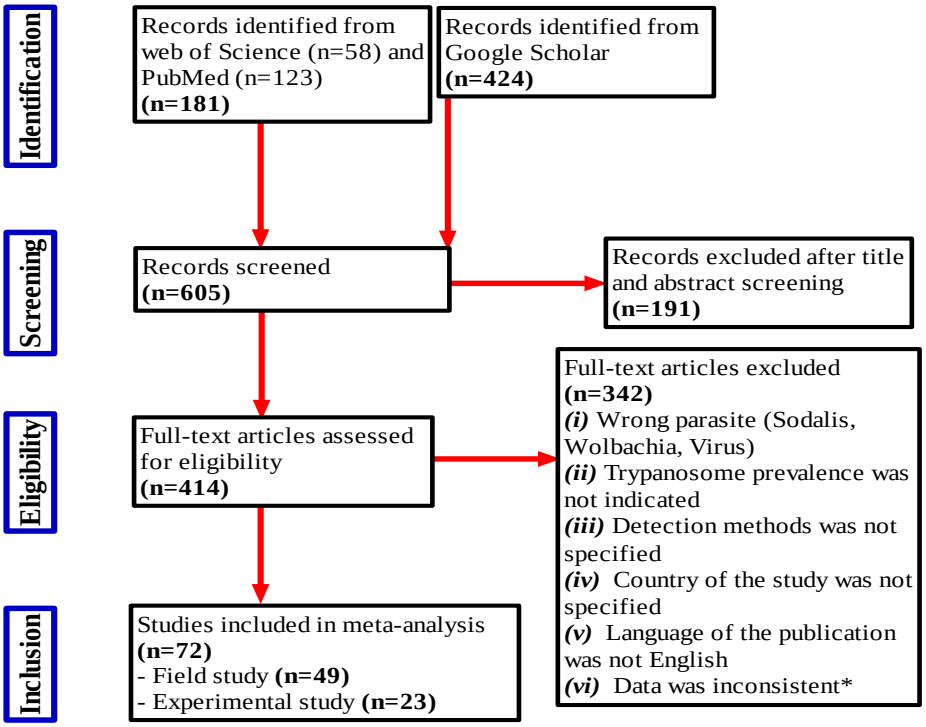

Figure 5.1. Flowchart detailing the number of studies excluded and included at each step for systematic review of the prevalence of trypanosome infection in tsetse flies. *data were inconsistent because total and/or positive tsetse flies were reported in an inconsistent way in the paper.

5.3.3. Diagnostic tools to assess trypanosome infection in a tsetse fly

The most widely used method constitutes the dissection of tsetse flies and microscopic evaluation of the organs. It is cheap (Lloyd and Johnson, 1924), but laborious, low in sensitivity and cannot differentiate mixed infections of trypanosome species and its different developmental stages in the fly (Ouma et al., 2000). The warm slide technique is occasionally used to assess the

prevalence of trypanosomes in tsetse flies. Accordingly, tsetse flies are allowed to salivate (probe) on a warm slide, then, trypanosome examination is done by microscopy of the slide (Burtt, 1946). An alternative for parasite detection with higher sensitivity is the inoculation of dissolved organ contents of tsetse flies in rats or mice for xenodiagnosis. Its added merit is that field isolates from mammals or tsetse flies can be collected via rodent inoculation for further studies. Its disadvantage is that diagnosis is not immediate and that *T. vivax* and *T. simiae* do not infect rodents (Gibson et al., 1999). Tissue culture techniques (in vitro cultivation) can be another option using different culture media. Cultivation is widely done for species of the *Trypanosoma brucei* group. The culture method is vital as it can provide information on pathogen viability and susceptibility to drugs (Gibson et al., 1999; Zongo et al., 2004). Isoenzyme band pattern examination technique is also possible. In this technique, 10-20 enzymes extracts from the trypanosome cytoplasm common to nearly all trypanosome species are separated by native electrophoresis and visualized by native staining. It requires a minimum of 100 million trypanosomes to test positive (Gashumba et al., 1988; Godfrey et al., 1990). The dot-ELISA test is another option which is based on the preparation of suspensions of different organs of tsetse flies that are applied on nitrocellulose membranes. Trypanosome species-specific monoclonal antibodies are used to detect the presence of trypanosomes in the suspension. The test is highly specific as monoclonal antibodies are used and it is simple, rapid, and can detect mixed infections via testing of one sample multiple times using different monoclonal antibodies (Bosompem et al., 1995 & 1996; Ouma et al., 2000). In the DNA probing technique, a denatured DNA sample (target) fixed on nitrocellulose is exposed to a radioactively labeled DNA-probe, which is a fragment of DNA of variable length. The probe - target complementary base pairing of the sequence in the probe is used to diagnose infection (Kukla et al., 1987; McNamara et al., 1989; Majiwa and Otieno, 1990). Conventional PCR has also been used (Gibson et al., 1999; Deborggraeve and Büscher, 2010). PCR works using either species-specific primers or generalist non-species-specific primers (e.g. ITS1) to differentiate trypanosomes. The advantage of ITS1 PCR is that only one test needs to be done to assess whether trypanosomes occur in the sample – regardless of the species, whereas in the species-specific PCR a sample must be tested repeatedly with each species-specific primer pair (Adams et al., 2006 & 2008). Trypanosome detection by PCR is done using the entire tsetse body or different tsetse organs and recently also even anal and oral droppings are used (Ravel et al., 2003; Ferreira et al., 2008). Another modern technique is the fluorescent fragment-length barcoding method (FFLB), which is a hybrid of PCR and sequencing. FFLB amplifies fragments with inter-species size variation by PCR using

fluorescently tagged primers, then, the sizes of the fragments of the PCR product are determined accurately using an automated DNA sequencer. Therefore, it discriminates trypanosome species by size polymorphisms. However, FFLB is too advanced and expensive for routine use in Africa (Hamilton et al., 2008; Adams et al., 2008 & 2010). Real-time PCR has the inherent ability to detect and quantify the number of trypanosomes in a sample (Ahmed et al., 2015). Finally, loop mediated isothermal amplification (LAMP) is a low-tech trypanosome detection technique. The target sequence is amplified by LAMP at a constant (isothermal) temperature of 60 - 65°C using either two or three sets of primers and a polymerase with high strand displacement activity in addition to a replication activity. Typically, 4 different primers are used to identify 6 distinct regions on the target gene, which leads typically to good specificity. The added advantage of the LAMP technique is that it does not require experience nor instruments except a water bath or incubator and results are obtained quickly (Kuboki et al., 2003).

5.4. Results

5.4.1. Inclusion of studies and data extraction

A total of 605 studies were initially screened of which 191 were excluded on the basis of their titles and abstracts. Of the remaining 414 which were fully evaluated, 72 were considered while 342 studies were excluded (Figure 5.1). The 72 selected articles involved 23 countries for a total of 236,740 tsetse flies checked for trypanosome infection. Of those 72 articles, 49 were reporting field studies with 202,182 tsetse flies analysed. The majority of the field studies (80%) used dissection (i.e. on 192,338 tsetse flies in total). The remaining 23 studies were laboratory experiments with 34,558 tsetse flies analysed of which 18 studies used dissection method. The studies included 12 different tsetse species. Samples analysed were saliva spit, anal droppings (diuresis fluid), midgut, proboscis, salivary glands and/or their DNA, DNA and pools of DNA from whole bodies. Methods used for the detection of trypanosomes were: dissection, microscopy of diuresis, probing on mice, warm slide probe, culture using media, isoenzyme analysis, DNA probing, Dot-ELISA, species specific PCR, ITS-1 PCR, real time PCR, species-specific LAMP and FFLB. Details are provided in Tables 5.1 and 5.2.

One third of the studies compared dissection positive results with at least one alternative serological or molecular technique: species-specific PCR (n=15), DNA probe (n=4), fluorescent fragment length barcoding (n=3), ITS-1 PCR (n=2), and dot-ELISA (n=1).

Table 5.1 List of published articles included in the systematic review for field studies with various method used for diagnosis of trypanosome infection.

Variable	No studies	No species	No flies	References
Overall field studies	**49**	**12***	**202182**	**[9,10,13,14,15,16,28,30,31,34,35, 36,40, 41-76]**
Country				
Angola	1	1	62	[50]
Burkina Faso	1	2	435	[40]
Cameroon	3	5*	6104	[13,53,73]
Democratic Republic of Congo	1	1	254	[72]
Equatorial Guinea	1	1	62	[36]
Ethiopia	1	4	384	[47]
Gambia	2	4*	3055	[31,57]
Ivory Coast	3	5	3707	[46,48,58]
Kenya	7	4*	41959	[14,41,28,30,67,74,75]
Liberia	1	3	2224	[68]
Nigeria	4	3	27502	[52,54,69,71]
Rwanda	1	3	5496	[61]
South Africa	3	2*	1323	[49,56,64]
Southern Sudan	1	1	117	[62]
Tanzania	10	4*	43923	[9,10,15,16,34,42,35,44,55,56]
Uganda	3	4	16350	[51,70,76]
Zambia	6	3*	49225	[43,45,53,60,66,68]
Detection method				
Culture media	1	3	1112	[58]
Dissection	39	12*	192338	[9,10,13,14,16, 28,30,31,35,40,41-48, 51,52,54,55,57-59,61,63-65,67-71,73-76]
Dot-ELISA	1	2	494	[28]
FFLB	1	*	91	[15]
ITS-1 PCR	2	1*	173	[15,55]
Sp. specific PCR	11	4*	7974	[36,49,50,53,56,60,62, 66,67,72,73]
***Glossina* sample type**				
DNA DO and Pool NDO	1	*	3638	[67]
DNA WB	4	3	1221	[50,53,60,62]
DNA WB & DO	1	1	279	[55]
Pool DNA WB	2	1*	312	[36,66]
Mid gut	8	5*	20792	[15,34,35,44,58,59,72,73]
MP	8	7*	46416	[9, 10, 16,30,49,52,54,64]
MS	1	2	1221	[46]
MPS	19	10*	73793	[13,28,31,40,42,57,60,61,64,65,67-71,74-76]
Proboscis	5	5*	46991	[43,14,45,56,63]
SG	1	1	7519	[41]
***Glossina* species**				
G. austeni	1	1	40	[64]
G. brevipalpis	6	1	5870	[10,45,51,56,61,64]
G. fuscipes	5	1	7071	[45,47,62,70,76]
G. longipennis	2	1	1305	[28,67]
G. medicorum	1	1	10	[48]
G. morsitans	10	1	51556	[10,31,40,45,47,52,57,61,68,71]
G. nigrofusca	3	1	294	[46,58,61]
G. pallicera	2	1	76	[58,59]
G. pallidipes	20	1	64395	[9,10,14,16, 28, 30,41,43, 44, 45,47,51,53,55,60,61,65,67,74,75]
G. palpalis	14	1	14669	[13,31,46,48,36,50,51,54, 58,59,63,69,72,73]
G. swynnertoni	5	1	14414	[9, 10, 16, 42, 44]
G. tachinoides	6	1	6367	[40, 47, 48, 54, 69, 71]
Mixed*	9	1	35865	[13,15,16,31,34,35,45,49,67]
Not determined	1	1	250	[66]

*= mixed tsetse sp. examined besides the indicated number of tsetse sp.; DNA DO and Pool NDO = DNA of dissected organs and of a pool of negative tsetse organs; DNA WB= DNA of whole fly body; DNA WB & DO = DNA of whole fly body and of dissected organs; Pool DNA WB = pooling DNA of whole fly body; MP = Mid gut and proboscis; MS = Mid gut and salivary gland; MPS = Mid gut, proboscis and salivary gland; SG, Salivary gland; SP, Salivary gland and proboscis.

Table 5.2 List of published articles included in the systematic review for laboratory experimental studies with various method used for diagnosis of trypanosome infection

Variable	No studies	No species	No flies	References
Overall experimental studies	**23**	**7***	**34558**	**[22,23,25,29,32,37,38,77-93]**
Country				
Belgium	3	2	1559	[25,81,84]
Burkina Faso	2	1*	1443	[37,83]
BFZ	1	2*	1092	[91]
France	1	1	594	[93]
Ghana	2	2	540	[29,82]
Kenya	5	7	19592	[22,32,77-79]
Tanzania	1	1	3274	[23]
Uganda	2	4	2011	[85,86]
United Kingdom	4	2	721	[38,87,90,92]
Zambia	1	1	1796	[88]
Zimbabwe	1	1	1936	[89]
Detection method				
Dissection	18	7*	22478	[22,25,37,77-79,81,83-91,93]
DNA probe	1	1	15	[32]
dot-ELISA	3	2	1240	[22,29,82]
Microscopy of diuresis	1	*	266	[91]
Sp. Specific PCR	2	3*	1246	[22,91]
probing on mice	1	1	300	[92]
Real Time PCR	1	1	150	[38]
warm slide probe	5	1*	8863	[23,78,89,90,91]
***Glossina* sample type**				
DF	1	*	532	[91]
Mid gut	5	2	2205	[29,32,38,88,90]
MP	3	2	1573	[81,84,91]
MS	2	2	1754	[23,89]
MPS	5	2*	4263	[22,25,82,83,87]
proboscis	3	6	12315	[77-79]
SS	5	1*	9011	[23,80,89,90,91]
SG	2	4	2411	[86,93]
SP	1	1	194	[85]
N.A	1	1	300	[92]
Glossina species				
G. austeni	1	1	1062	[79]
G. brevipalpis	2	1	1256	[79,86]
G. fuscipes	2	1	1570	[79,86]
G. morsitans	19	1	22607	[23,25,29,32,38,77-79,81,82,85-88,89,90,91-93]
G. pallidipes	4	1	2619	[22,82,86,90]
G. palpalis	4	1	2184	[37,79,84,91]
G. tachinoides	1	1	1009	[79]
Mixed*			2251	[83,91]

*= mixed tsetse sp. examined besides the indicated number of tsetse sp.; DF = Diuresis fluid; MP = Mid gut and proboscis; MS = Mid gut and salivary gland; MPS = Mid gut, proboscis and salivary gland; SS = Saliva spit; SG = Salivary gland; SP = Salivary gland and proboscis; N.A. = Not available

5.4.2. Meta-analysis of dissection based field studies

The overall trypanosome prevalence of flies in the field studies (n=39) was 10.3% (95% CI = 8.1, 12.4) (Figure 5.2). Significant between-study heterogeneity was observed (P<0.001). Different risk factors were thus further analysed. Results are presented in Table 5.3. The prevalence of

trypanosomes decreases with publication year (*P*=0.035). Trypanosome prevalence differs significantly between countries (*P*=0.004). The prevalence ranged from 4.1% in Rwanda to 40.5% in Burkina Faso. The type of tsetse fly sample (body part) did not have a significant effect on the prevalence (*P*=0.2155). The prevalence of trypanosomes ranged from 6.5% in midguts to 30.8% in the pooled midgut /salivary glands samples. Tsetse fly species or group (*morsitans*, *fusca*, *palpalis*) were not significant risk factors (*P*=0.1466). The highest trypanosome prevalence was observed in *G. nigrofusca* (26%, n=3, *fusca* group) and the lowest was observed in *G. longipennis* (0.2%, n=2, *fusca* group). The two variables that were significant in the univariate analysis (Table 5.3) remained significant with minor changes in the estimates of the odds ratios in the multivariate meta-regression analysis (results not shown).

5.4.3. Meta-analysis of dissection based laboratory experiments

The overall trypanosome prevalence of flies in the laboratory experiments (n= 18) was 31.0% (95% CI = 20.0, 42.0) (Figure 5.3). Significant between-study heterogeneity was also observed in these studies (P<0.001). However, the trypanosome prevalence did not differ significantly between countries (P = 0.0916) nor as a function of the publication year (P=0.184) (Table 5.4). No significant (P=0.9545) differences in trypanosome prevalence among the seven tsetse species were observed. The sample type (body part) was significantly (P=0.0122) associated with trypanosome prevalence. The highest trypanosome prevalence was observed in the proboscis (Table 5.4).

Table 5.3 Univariate meta-regression analysis of risk factors for the prevalence of trypanosome infection based on the field studies with dissection method used for diagnosis of trypanosome infection.

Variables	**Prevalence**			**Odds ratio**			
	Estimate	**95% CI**		**Estimate**	**95% CI**		**p-value**
Year of publication	-		-	0.998	0.996	0.999	0.035
Country							
Burkina Faso	40.5	28.1	53.0	Ref.			
Ivory Coast	18.6	13.7	23.6	0.82	0.69	0.97	0.021
Nigeria	18.0	5.0	30.9	0.80	0.67	0.95	0.010
Cameroon	10.9	2.9	18.8	0.74	0.62	0.89	0.002
Uganda	10.0	3.0	17.0	0.74	0.62	0.88	0.001
Zambia	8.6	3.1	14.1	0.73	0.61	0.86	<0.001
Liberia	8.2	4.4	12.1	0.74	0.61	0.89	0.002
Kenya	7.4	3.5	11.4	0.72	0.61	0.85	<0.001
Tanzania	7.0	5.2	8.9	0.71	0.61	0.84	<0.001
South Africa	6.8	0.2	20.5	0.71	0.58	0.88	0.002
Gambia	6.0	5.2	6.8	0.71	0.59	0.85	<0.001
Ethiopia	4.9	0.6	9.2	0.70	0.58	0.84	<0.001
Rwanda	4.1	3.0	5.1	0.70	0.58	0.84	<0.001
***Glossina* sample type**							
MS	30.8	7.6	53.9	Ref.			
Salivary glands	19.3	18.4	20.2	0.91	0.69	1.19	0.472
MPS	11.2	8.5	14.0	0.84	0.72	0.98	0.023
Proboscis	10.6	4.3	16.9	0.83	0.70	0.98	0.029
Midgut	6.5	4.6	8.3	0.83	0.70	0.98	0.027
Midgut and proboscis	6.5	5.0	8.0	0.80	0.68	0.94	0.007
***Glossina* species**							
G. nigrofusca	25.9	1.9	49.9	Ref.			
G. pallicera	23.5	12.4	34.7	1.04	0.81	1.34	0.751
G. medicorum	20.0	4.8	44.8	0.96	0.66	1.39	0.811
G. tachinoides	17.7	8.5	26.9	0.93	0.78	1.12	0.454
G. morsitans	15.8	9.0	22.6	0.92	0.77	1.08	0.304
G. austeni	15.0	3.9	2.16	0.91	0.68	1.22	0.520
G. palpalis	10.5	6.4	14.6	0.87	0.74	1.03	0.107
G. mixed species	9.6	4.3	14.8	0.86	0.72	1.03	0.102
G. swynnertoni	9.2	3.0	15.4	0.86	0.71	1.03	0.101
G. pallidipes	8.3	5.8	10.8	0.85	0.72	1.00	0.052
G. brevipalpis	5.8	2.3	9.3	0.83	0.69	1.00	0.046
G. fuscipes	1.1	0.1	2.1	0.79	0.65	0.96	0.017
G. longipennis	0.2	0.1	0.5	0.78	0.63	0.98	0.031

MS = Mid gut and salivary glands; MPS = Mid gut, proboscis and salivary glands.

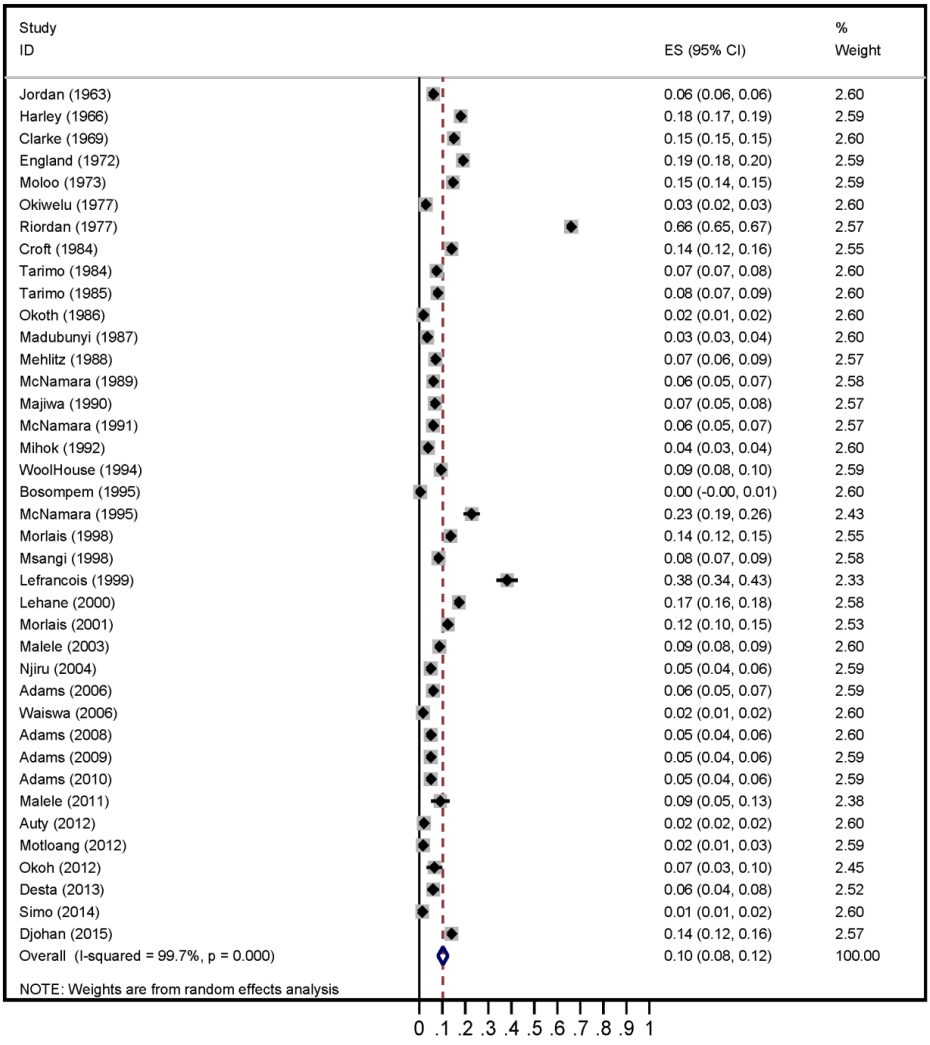

Figure 5.2. Forest plot of the prevalence of trypanosome infection in tsetse flies by the dissection method for field studies. The forest plot denotes the 95% confidence interval of the infection prevalence for each study separately, together with the overall estimate on the last line.

Table 5.4 Univariate meta-regression analysis of risk factors for the prevalence of trypanosome infection based on the laboratory experimental studies with dissection method used for diagnosis of trypanosome infection.

	Prevalence (%)			Odds ratio			
Variables	**Estimate**	**95% CI**		**Estimate**	**95% CI**		**p-value**
Year of publication	-		-	0.996	0.99	1.00	0.184
Country							
Zimbabwe	54.5	52.2	56.8	Ref			
Kenya	50.9	31.7	70.0	0.96	0.62	1.51	0.866
Zambia	44.3	42.0	46.6	0.90	0.49	1.65	0.726
Uganda	21.4	14.9	27.9	0.71	0.45	1.15	0.155
Belgium	20.8	13.9	27.8	0.72	0.44	1.18	0.181
United Kingdom	20.5	0.0	41.3	0.71	0.43	1.17	0.171
Burkina Faso and Zimbabwe	20.1	9.1	31.0	0.71	0.42	1.22	0.192
France	14.5	11.6	17.3	0.67	0.37	1.22	0.181
Burkina Faso	10.0	8.4	11.5	0.64	0.38	1.08	0.093
***Glossina* sample type**							
Proboscis	56.1	43.3	68.8	Ref			
Midgut and salivary gland	32.7	0.0	76.3	0.80	0.59	1.09	0.145
Salivary gland	22.3	16.8	27.8	0.71	0.58	0.89	0.004
Midgut and proboscis	20.2	13.5	26.9	0.70	0.56	0.89	0.005
Midgut	19.0	0.0	51.4	0.69	0.53	0.89	0.007
MPS	17.6	10.1	25.1	0.71	0.56	0.89	0.006
Salivary gland and proboscis	9.8	5.6	14.0	0.63	0.42	0.95	0.028
***Glossina* species**							
G. brevipalpis	51.9	0.0	100.0	Ref			
G. tachinoides	40.2	37.2	43.3	0.89	0.48	1.66	0.698
G. morsitans	37.9	23.6	52.2	0.87	0.59	1.28	0.455
G. fuscipes	31.7	11.2	52.2	0.82	0.49	1.36	0.415
G. austeni	31.5	28.7	34.2	0.81	0.44	1.52	0.499
G. palpalis	31.0	23.4	38.7	0.796	0.52	1.22	0.278
G. pallidipes	11.9	0.0	24.0	0.67	0.42	1.07	0.088
Mixed	10.0	8.4	11.5	0.66	0.35	1.22	0.174

MPS = Mid gut, proboscis and salivary gland

5.4.4. Sensitivity of advanced detection methods

The results of the meta-analysis of the 25 studies using alternative diagnostic tests on dissection positive samples are shown in Figure 5.4. With the exception of the dot-ELISA (sensitivity of 91%, which was represented only by one study), the remaining methods had similar levels of sensitivity ranging from 43% for DNA probe to 62% for fluorescent fragment length barcoding.

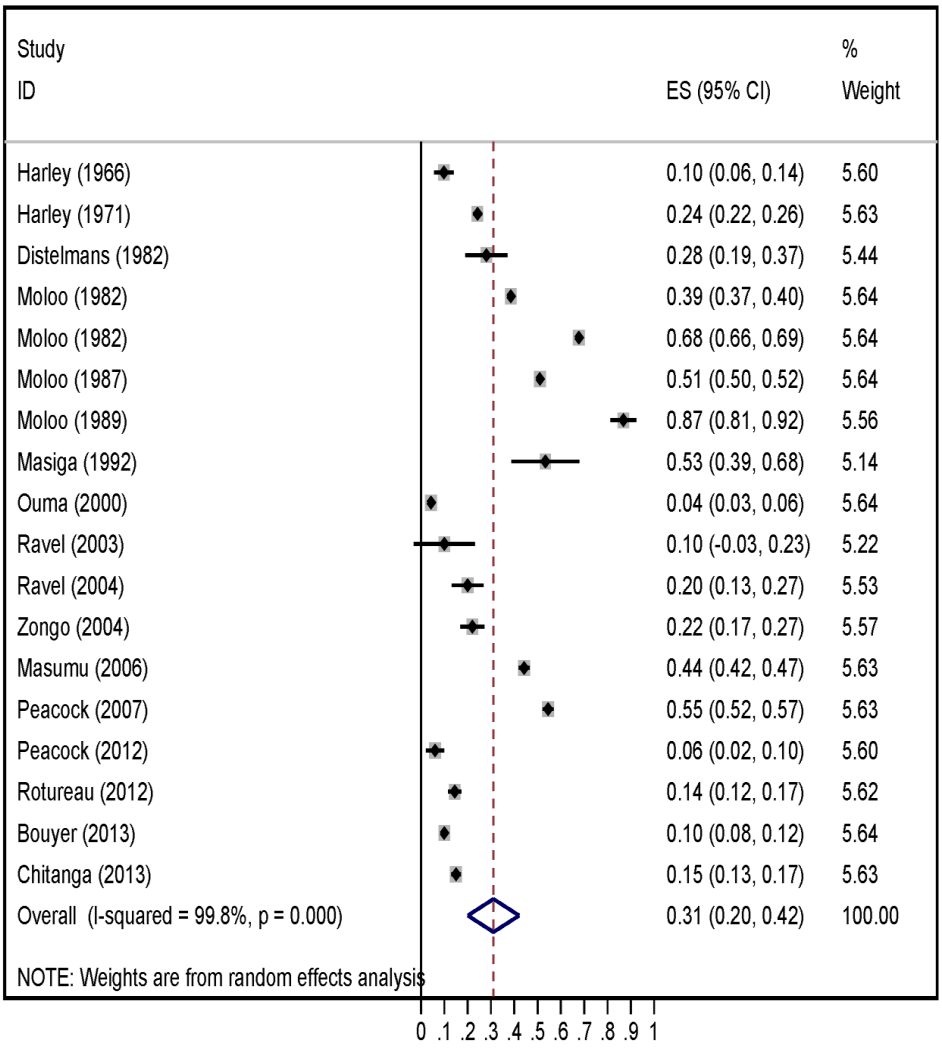

Figure 5.3. Forest plot of the prevalence of trypanosome infection in tsetse flies by the dissection method for laboratory experiments. The forest plot denotes the 95% confidence interval of the infection prevalence for each study separately, together with the overall estimate on the last line.

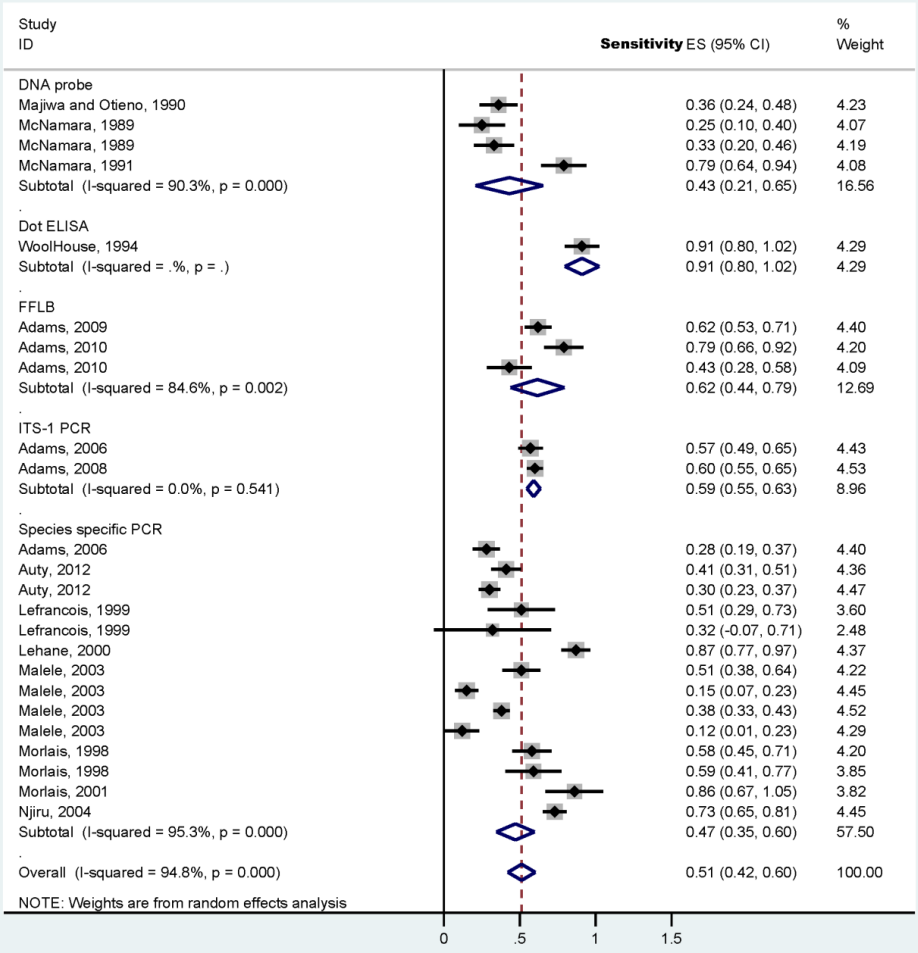

Figure 5.4. Forest plot for the sensitivity of molecular and serological detection methods of trypanosome infection in the tsetse flies using the dissection method as gold standard. The forest plot denotes the 95% confidence interval of the sensitivity for each study separately, together with the overall estimate on the last line.

5.5. Discussion

The scientific literature on trypanosome detection methods and prevalence in tsetse flies published in English since more than a half century covering natural and experimental infections of tsetse flies was reviewed in this paper.

As expected, the prevalence of trypanosome infection in tsetse flies is higher in laboratory experiments than in field collections of tsetse flies with an overall prevalence of 31% and 10% respectively. In laboratory experiments, blood meals and external conditions are standardized and all feeding flies ingest parasites. In field collected tsetse flies, the prevalence of trypanosome infected hosts and their parasitemia will be the determining factors explaining the prevalence in flies.

From our meta-analysis, it appears that differences in prevalence exist in field collected tsetse flies according to year and country. The factor "country" should be interpreted with care as many factors can explain the differences between countries, e.g., the ecological context and national vector control measures. If our review allowed pinpointing spatial differences, it could not clearly bring a precise explanation of the variations in infection prevalence.

The negative relationship between the year of publication and the prevalence of infection is a paradox. Encroachment, i.e., the degrading effect of human activities on the environment, has taken place in most regions of sub-Saharan Africa. Encroachment causes a decrease of the tsetse fly population size. However, as counterbalancing effect, the prevalence of the trypanosome infection of the flies has been observed to increase allowing for persistent transmission even when the tsetse fly vectors are scarce (Ducheyne et al., 2009; Mweempwa et al., 2015). However, the opposite effect is observed. This might be due to the higher intensity of drug treatment of the livestock by the farmers. Indeed, most farmers in endemic areas treat their herds regularly using trypanocidal drugs (Grace et al., 2009). It is known that prolonged and persistent use of trypanocidal drugs in the field decreases/disrupts the transmission of trypanosomes by the tsetse flies (Lutumba et al., 2005; Hargrove et al., 2012) thus reducing the risk of tsetse infection.

Our last objective was to assess the sensitivity of various diagnostic methods for the detection of trypanosomes in the tsetse flies using the dissection method as gold standard. The sensitivity of molecular/serological tests that were performed on positive samples (i.e. by dissection) was only around 50%. The alternative diagnostic tools applied to the dissection positive samples were thus characterised by low sensitivity, and no information on specificity is available at all. The

currently available molecular and serological techniques are developed and optimized for trypanosome detection in the host; their detection performance in the insect (tsetse fly) is a different story. This study revealed that the tests apparently work suboptimal for tsetse fly samples. Conditions and specimens used are not standardized or externally controlled for detection of trypanosomes in tsetse flies. Comparing several tests on the same specimen panel would allow more accurate comparisons of the sensitivity and specificity.

5.6. Conclusions

Dissection remains the gold standard for the determination of the infection status of tsetse flies. Alternative molecular and serological techniques have currently too low sensitivity and their specificity is unknown, which warrants further investigation before they can be employed on a routine basis. Both temporal and spatial variation in trypanosome infection prevalence of field collected tsetse flies exists, but it needs to be investigated further how this variation can be explained.

5.7. Acknowledgments

This work was financially supported by the VLIR-UOS South Initiative. We would like to thank Dr Olana Merera, Dr Megersa Bedasa, and Dr Biyansa Adugna for their critical comments and editing the retrieved data.

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Chapter 6:

General Discussion

HAT puts 60 million people at risk every year and AAT costs more than 4.5 million USD to Africa every year. Eradication of trypanosomosis would lead to a threefold increase of the cattle population in Africa (Murray and Gray, 1984; Wint and Rogers, 2000). Thus, African heads of state decided in 2000 to eliminate tsetse flies from Africa via the Pan African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC) initiative. Ethiopia, Mali, Burkina Faso, Botswana, Kenya, Uganda, and Tanzania have begun to implement the plan in selected areas of their countries (Kabayo, 2002).

Tsetse flies infest a vast area (~10 million km²) located in sub-Saharan Africa. However, the distribution of tsetse flies in this area is not continuous. Tsetse flies occur in dynamic pockets, many of which are relatively isolated in space and time. The interruption can be attributed to fragmentation of the vegetation cover, either occurring naturally or made by man or to variations in the altitude as regions above 1800m are not suitable for tsetse flies (Jordan, 1993). As a general rule, environmental factors such as vegetation cover, land use pattern, land cover type, humidity, normalized difference vegetation index (greenness of vegetation), temperature, rainfall and elevation are important determinants of the occurrence, abundance and distribution of tsetse flies. Spatial heterogeneity in environmental conditions results in local differences in tsetse fly abundance and trypanosome transmission dynamics (Kitron et al., 1996). All environmental and ecological factors affecting tsetse fly abundance and trypanosome infection prevalence are therefore essential to consider when planning, implementing and evaluating vector control and elimination strategies.

6.1. The spatial distribution of tsetse flies and trypanosomosis in Ethiopia

In Ethiopia, the last country-wide census on tsetse flies and trypanosomosis was conducted more than 30 years ago (Langridge, 1976). Over the last three decades, dynamic changes have been observed in tsetse fly infested areas of Ethiopia particularly related to ecology, climate, land use and intensive settlement of livestock and human population (Reid et al., 2000). This indicates that the fight against tsetse flies has long been intensified at several fronts in Ethiopia, but the information on tsetse fly abundance and distribution has not been updated accordingly. Five tsetse fly species, namely, *G. pallidipes*, *G. m. submorsitans*, *G. fuscipes*, *G. tachnoides* and *G. longipennis* (Langridge, 1976; Jordan, 1993) have been involved in the transmission of *T. congolense*, *T. vivax* and *T. brucei*. Of 1.1 million km² of land area, about 240,000 km² of fertile lands are under threat of trypanosomosis. Therefore, vegetation clearing, insecticides, chemotherapy, and settlement (or resettlement) have been recommended as the most effective and feasible tsetse control methods (Habtemariam et al., 1983). Subsequently, massive deforestation (resettlement and domestic animal development programs) took place in Gambella after 1985 (Nigatu et al. , 1992; Hadis et al., 1995), followed by pour-on application (Leak et al., 1995) and insecticide-impregnated targets at Gibe (Leak et al., 1996), biconical and NGU traps at upper Didessa (Belete et al., 2004) and Nzi, epsilon, pyramidal, NG2G, biconical and canopy traps at Chanka in west Wollega in 1997 (Mihok et al., 2007). In 1996, a tsetse mass rearing factory has been built in Kaliti, Ethiopia, to deploy the release of sterile male technique to eliminate tsetse flies from the southern rift valley (Alemu et al., 2007).

A new country-wide survey in this thesis indicated that there is a widespread distribution of four tsetse fly species (*G. pallidipes*, *G .m. submorsitans*, *G. fuscipes* and *G. tachnoides*) in Ethiopia. Two distinct distribution patterns of tsetse fly species in Ethiopia can be discerned: *(i)* the occurrence of only one tsetse species (*G. tachnoides* in Amhara region and. *G. m. submorsitans* in Benishangul-Gumuz region), and *(ii)* the occurrence of more than one tsetse fly species (Oromia region). Multiple tsetse fly species are recorded in the same area and occur even together in one and the same trap in a number of locations of Oromia region. Such species overlap reflects the heterogeneity of the ecological area which can thus support diverse tsetse fly species, hence aggravating the transmission of trypanosomes as vectors are more abundant. Co-existence of multiple tsetse fly species in one niche is made possible by avoiding deleterious

competition: *(i)* peak of the diurnal activity differs. Savannah species, such as *G. morsitans* and *G. pallidipes*, peak in the morning and late afternoon (Crump and Brady, 1979; Hargrove and Brady, 1992), the riverine species such as *G. palpalis* and *G. fuscipes* peak in the middle of the day (Crump and Brady, 1979; Mohamed-Ahmed and Odulaja, 1997), *(ii)* difference in seasonal population peaks, *(iii)* difference in host species preference – *G. morsitans* prefers warthog, bushpig and bovidae whilst *G. palpalis* is opportunistic (Weitz, 1963, Challier, 1982; Clausen et al. 1998), *(iv)* difference in body site preference on a host - 80% of *G.pallidipes* probes on the leg of ox whilst *G. morsitans* probes the entire body, *G. tachnoides* probes below the knee of man whilst *G. palpalis* probes above the waist (Jordan, 1993; Torr et al., 2007).

In the current survey, tsetse flies are more abundant in the south-western part of Ethiopia where tsetse fly densities vary substantially in space due to the landscape attributes as reported elsewhere (Lambin et al., 2010). The tsetse fly abundance is correlated inversely with altitude. Tsetse flies are more abundant in the lowlands (<1200m) and therefore these regions should be the focus of intervention. Particularly important are low lying areas with annual temperature between 19-30°C (Matawa et al., 2013). However, local variations occur. There are areas with no, low, moderate to higher tsetse fly abundance. Peasant associations located near wildlife reserve (Abuna Gali PA near Didessa wildlife reserve) have more tsetse flies despite their higher altitude (>1500m). Tsetse flies expand abundantly in protected game reserves and national parks of the tsetse-endemic area and tsetse flies migrate following the seasonal mobility of the wildlife in search of water (Munang'andu et al., 2012). This warrants site-specific strategies for control and surveillance, and achieved progress should be monitored continuously to arrive eventually at elimination of AAT. The tsetse fly abundance is also correlated positively with the size of the drainage system. Hydrologic networks (wetlands) host the highest number of tsetse flies in Ethiopia. River size, width, depth, and flow affect the type of vegetation along river banks and the associated shade, temperature and humidity which, in turn, affect tsetse fly abundance (Bouyer et al., 2005). Given that tsetse couldn't able to live without blood meal, the drainage system also determines the size and diversity of hosts which is essential for the tsetse fly as it feeds exclusively on blood. Transmission of the trypanosomoses would be expected to be high in those wetland areas, therefore, these areas seem the most likely to maintain enzootic cycles. Therefore, the drainage systems determines vegetation type and the inhabitant host animals. No significant variation in tsetse fly abundances between years was observed in the survey, revealing that tsetse fly population remained stable over the study years. Thus, it seems that the on-going

control efforts did not suppress the tsetse flies although the study have not covered all seasons to decipher population dynamics of tsetse.

The current survey also revealed that cattle trypanosomosis is widespread in tsetse fly-invaded regions with an overall prevalence of 9.6% where 76% is attributed to *T. congolense*, 18% to *T. vivax*, 3.6% to *T. brucei* and 2.4% to mixed infections of *T. congolense* and *T. vivax*. Cattle trypanosomosis is more abundant in lower altitudes (<1200m) and larger size drainage systems which is in line with the distribution of tsetse fly abundance previously explained. The study indicated that male animals and older animals are infected more often than females and young animals. The high prevalence among older adult cattle groups, particularly males, can be explained by *(i)* their higher attractiveness to flies (Torr et al., 2001; Torr et al., 2007b), *(ii)* the higher stress linked to their use as draft animals and *(iii)* the malnourishment of young males used prematurely for traction work (Simukoko et al., 2007). In the study areas significant prevalence variation has been observed among the studied 82 PAs: 0% in 12 PAs, 1-5% in 17 PAs, 5-10% in 28 PAs, 11-15% in 15 PAs and 15-31% in 8 PAs. Thus both cattle trypanosomosis and tsetse fly abundance have been found to vary substantially at various localities (PAs). This is partially due to variation in encroachment i.e. the degrading effect of human activities on the environment causing at first a decrease of the tsetse fly population size. However, as counterbalancing effect, it has been observed that the trypanosome infection prevalence of the tsetse fly increases allowing for persistent transmission even when vectors are scarce (Ducheyne et al., 2009; Mweempwa et al., 2015).

Surprisingly, no relationship has been found between tsetse fly abundance and trypanosomosis prevalence in this new survey. It is essential to investigate the lack of such a relationship in more detail, as it might indicate that trypanosome transmission in the area is shifting from the tsetse fly vector to other vectors that can transmit the trypanosome mechanically.

Overall, this study revealed that tsetse and trypanosomosis is still abundant and widely distributed in Ethiopia despite decades of control and eradication efforts. The efforts done so far have therefore not been successful to control neither the vector nor the parasite, and different causes of this failure have been given. It has been reported that tsetse flies have advanced to previously un-invaded new locations (Leak et al., 1993). Furthermore, multi-drug resistant trypanosomes occur widely in the Gibe valley (Leak et al., 1996; Mulugeta et al., 1997; Rowlands et al., 2001; Moti et al., 2012), in north western Ethiopia (Afewerk et al., 200), in north Omo valley (Assefa and Abebe, 2001) and in upper Didessa valley (Tewelde et al., 2004).

Due to mechanical transmission of the trypanosome by other biting flies, trypanosome transmission might have continued, even in the absence of tsetse flies (Desquesnes and Dia, 2003; Desquesnes and Dia, 2004; Cherenet et al., 2006; Sinshaw et al., 2006; Fikru et al., 2012). The failure can also be brought back to human factors. Control interventions have been fragmented and uncoordinated and long-term coherent policies are largely absent (Brightwell et al., 2001), and no or little information is given to the farmers. Accurate data on vector and parasite distributions as well as on risk factors for trypanosomosis among the domestic hosts are of paramount importance to control the disease with the ultimate goal of achieving eradication.

6.2. Development of anti-tsetse saliva ELISA test to monitor tsetse flies

During control interventions, AAT and tsetse fly densities could decrease to such a low level that the disease is no longer diagnosed in a survey of individual animals and that tsetse flies are no longer caught in the traps while tsetse flies are still roaming around sporadically and biting animals infrequently. Traps become inefficient in trapping at low tsetse density (Dicko et al., 2014). With the objectives of PATTEC, i.e., to eliminate tsetse flies from Africa, traps may not be the appropriate tool *(i)* to monitor the impact of vector control programs and assess the reduction of cattle-tsetse fly contact, *(ii)* to evaluate the efficiency of protective barriers, *(iii)* to detect early re-invasion by tsetse flies of previously cleared areas and *(iv)* to estimate the risk of developing the disease in animals exposed to tsetse fly bites during epidemiological studies. Consequently, a novel diagnostic tool that is able to detect whether individual animals in herds or localities were exposed to tsetse flies is required to monitor a targeted intervention and, if needed, to give an early warning when the intervention is not fully efficient.

Therefore, a novel diagnostic tool was developed with the aim to assess whether and when and to what extent an individual animal has been bitten by tsetse flies. The tool is based on an indirect ELISA, and has first been successfully developed for mice and pig. The ELISA uses either total tsetse saliva or the recombinant Tsal1 protein of the tsetse fly as coating antigen. Both ELISAs provide the following information. They *(i)* detect the exposure of the animal to tsetse fly bites, *(ii)* differentiate between varying levels of tsetse fly challenge (no, low and high exposure), *(iii)* estimate the length of exposure to tsetse fly bites based on the persistence and dynamics of induced antibodies, *(iv)* detect the bite of newly arriving tsetse flies after long periods of non-exposure (tsetse fly re-invasion), *(v)* detect presence of any species of tsetse flies (test is not tsetse species specific). Therefore, the currently developed ELISAs are useful tools in the sero-

epidemiological (prevalence) study of tsetse flies, especially for regular monitoring of tsetse fly exposure and to assess the efficiency of implemented or on-going tsetse fly control activities (Fontaine et al., 2011).

However, both ELISAs need to be further improved. They *(i)* are only optimized for mice and pig but not for cattle which is the most important livestock of Africa, for still unknown reason, sera of cattle give high backgrounds hiding specific bindings *(ii)* have low specificity due to cross-reactivity with other biting insects (*Tabanus* and *stomoxys* sp.), *(iii)* have low test sensitivity in separating naïve from low exposure in pigs, *(iv)* require *E.coli* lysate inclusion in porcine sera (prior sample treatment) to increase test specificity to clear out the unspecific reactants from the sera, *(v)* demand serum dilution to 1:1600 implying extra costs, time and labor, and *(vi)* require horseradish peroxidase (HRP enzyme) conjugated secondary host species-specific antibodies for each livestock species to detect the presence of tsetse fly induced antibodies in their test sera which again inflates the costs of the test. To overcome the above drawbacks, another approach has been attempted in this study based on the competitive/inhibition ELISA test in the presence of nanobodies. The competitive ELISAs also use total tsetse saliva or recombinant Tsal1 protein of tsetse flies as coating antigen. Antibodies in the test serum and the nanobodies against tsetse saliva protein compete for binding onto the coating antigen. The test serum is considered positive when the binding of nanobodies is inhibited. An important advantage of the competitive/inhibition ELISA test as compared to the previously developed ELISA test is that it does not require animal species specific secondary conjugated antibodies. Unfortunately, many problems were encountered with this approach and it was not successful to develop a sufficiently specific and sensitive test. The competitive nanobody approach works for mice and pig (Caljon et al., 2015).

Generally, the current total tsetse saliva based ELISA test was not tsetse species specific. The detection of host antibodies raised against whole saliva extracts has been reported for several tsetse species: *G. m. morsitans* to assess human exposure in East Africa (Caljon et al., 2006); *G. f. fuscipes* to assess human exposure in Central Africa (Poinsignon et al., 2007 & 2008); *G. p. gambiensis* to assess human exposure in West Africa (Dama et al., 2013); *G. m. submorsitans* to assess cattle exposure in West Africa (Somda et al., 2013 & 2016). The findings of different studies showed that proteins from the Tsal family are major constituents of tsetse saliva, and induce strong antibody responses in tsetse exposed hosts. These proteins were thus considered as interesting candidates to develop biomarkers of tsetse exposure. In this thesis, the rTsal ELISA

test derived from *G. m.morsitans* detects antibodies raised by other tsetse species. The Tsal specific indirect ELISA (Caljon et al., 2014) and competitive ELISA using nanobody families (family III, TsalNb-05 and TsalNb-11) were able to detect exposure to a broad range of tsetse species such as *G. m. morsitans*, *G. pallidipes*, *G. p. gambiensis* and *G. fuscipes* and did not cross-react with the other hematophagous insects (*Stomoxys calcitrans* and *Tabanus yao*) in mice and pig (Caljon et al., 2015). A nanobody-based competitive immunoassay to detect anti-Tsal antibodies is interesting as it can be used for testing any mammal sera without the need for specific conjugated anti-mammal antibodies (Caljon et al., 2015). Similarly, another study revealed that in *G. m. morsitans*, Sgp3, Tsal and Antigen 5 proteins have higher immunogenicity than the other abundant proteins, such as 5' nucleotidase and TSGF family whereas in *G. fuscipes*, proteins that belong to the adenosine deaminase-related growth factors (ADGF) family display the highest immunogenicity potential in mice and cattle. According to this work tsetse flies in the same subgenus displays greater similarity and cross reactivity in immune response (Zhao et al., 2015). The multiple biomarkers available appear to detect a range of tsetse species. Thus the five tsetse species (*G. pallidipes, G. fuscipes, G.m.submorsitans, G.tachinoides* and *G.longipenis)* present in Ethiopia could be detected using some of the available novel serological salivary biomarkers, but most of these biomarker assays are yet only tested under controlled laboratory conditions.

In this study, specific antibody response to total and rTsal1 has not been observed in calves experimentally exposed to high and low *G. pallidipes* bites in Ethiopia. However, in West Africa cows exhibited strong antibody responses against total saliva extracts of *G. m. submorsitans* following high and low level of exposure experimentally. Surprisingly, the same calves which were exposed to 50 *G. m. submorsitans* flies twice a week during 11 weeks (high exposure group) didn't show detectable antibodies to a synthetic $Tsal1_{52–75}$, but for the low exposure group (10 flies weekly during the same period) antibodies were detected (Somda et al., 2016). This suggests that saliva proteins of different tsetse species could vary in their immunogenicity as witnessed by studies in Ethiopia and West Africa. The lack of antibody response to rTsal1 might suggest that a single epitope behaves differently in terms of immunogenicity according to the exposure conditions. The mechanisms underlying this intriguing result are not yet understood but could be related to antigen specific B cell exhaustion or anergy induced by high antigenic stimulation levels.

6.3. Prevalence of trypanosome infection in tsetse flies

The distribution of the prevalence of tsetse flies is mostly influenced by spatial factors such as altitude (climate), drainage system (host and vegetation) and by local factors at peasant association level, such as the presence of game reserves and land utilization/encroachment. These factors influence the prevalence and incidence of trypanosomosis as reported in Chapter 3 of this thesis. The trypanosomes travel from host to vector and from vector to host, and it can be assumed that an increased trypanosome abundance in one is having a profound effect on the trypanosome abundance in the other (Rogers, 1988). Trypanosome infections in tsetse flies are gained proportional to the infection rate or parasitemia in the hosts. Tsetse flies are thought to remain infected once acquiring a trypanosome infection and the trypanosome infection prevalence in the tsetse fly population can thus only decrease if the infected tsetse flies are removed either through the natural death or through control efforts, and when naïve tsetse flies are not infected or their infection is delayed. Hosts are assumed to lose trypanosome infections through natural recovery, treatment or death (Rogers, 1988; McDermott and Coleman, 2001). The risking of being infected with trypanosomes for livestock (also called AAT risk) is a function of three factors: *(i)* abundance of tsetse flies (which can be estimated by trap catches), *(ii)* the proportion of animals in the tsetse fly blood meals that correspond to livestock (as compared to for instance wildlife) and *(iii)* the proportion of tsetse flies infected with trypanosomes (Snow and Tarimo, 1983; Leak et al., 1993). Other authors argue that the transmission of trypanosomes from tsetse fly to host is a function of tsetse fly biting rates, proportion of tsetse flies infected, proportion of tsetse fly bites resulting in a host infection and the ratio of vectors to hosts (McDermott and Coleman, 2001). Therefore, the importance of tsetse flies as a vector is not only determined by its abundance (Chapter 3 of this thesis), but also by its ability to become infected and transmit pathogens (Leak, 1999). A given tsetse fly species can be a major vector of trypanosomes in a region posing a great risk, despite its relatively low population density, if it is an efficient transmitter (Motloang et al., 2012). Furthermore, trypanosome infection prevalence in the tsetse fly is one of the fundamental components of AAT risk analysis in estimating the risk of an individual animal to acquire a trypanosome infection (Rogers, 1988; McDermott and Coleman, 2001). Therefore, a systematic review of the trypanosome infection prevalence in tsetse flies was conducted in this thesis (Chapter 5).

The overall prevalence of trypanosome infection in tsetse fly populations was 10.3% and 31.0% for the field survey data and laboratory experiment data, respectively. Both spatial (country) and

temporal (year of publication) variation are noticed in trypanosome infection prevalence of field collected tsetse flies. Such spatio-temporal heterogeneity is due to the interplay of many different factors. One of the main factors is the disturbance of the host and tsetse fly environment which varies across space and time. Furthermore, it is known that prolonged and persistent use of trypanocidal drugs in the field decreases/disrupts the transmission of trypanosomes by the tsetse flies (Lutumba et al., 2005; Hargrove et al., 2012) thus reducing the risk of tsetse fly infection. Similarly, vector control interventions leading to the death of infected tsetse flies decrease the tsetse fly abundance and thus the risk of being bitten by a tsetse fly for the cattle (Hargrove et al., 2012). However, as counterbalancing effect, the prevalence of the trypanosome infection of the flies has been observed to increase allowing for persistent transmission even when the tsetse fly vectors are scarce. Such increase is manifested in tsetse flies under "stress" conditions due to encroachment (Ducheyne et al., 2009; Dicko et al., 2015; Mweempwa et al., 2015), shifts in climate/increased temperature (Dicko et al., 2015) and nutritional stress/starvation (Kubi et al., 2006; Akoda et al., 2009) and these tsetse stressors are common under natural conditions. Therefore, spatial and temporal changes in the environment and human activity lead to variations in both the abundance and trypanosome infection prevalence of tsetse fly populations with heterogeneous risk of cyclical transmission both in space and time. However, we should not also forget another scenario that in sites with the highest fragmentation *Glossina morsitans* will disappear (Mweempwa et al., 2015).

Dissection of the tsetse fly to diagnose its trypanosome infection status is by far still the most applied technique in field studies or monitoring activities. Diagnosis of the trypanosome based on molecular techniques has been shown to have high sensitivity and specificity in the mammalian host (Deborggraeve and Büscher, 2010). A spectrum of more advanced techniques, ranging from DNA probe to different PCR formats such as PCRs and real-time PCRs with different targets on the parasite DNA (Gibson et al., 1999) and LAMP (Kuboki et al., 2003) have also been used to detect the presence of trypanosomes in the tsetse fly. However, the sensitivity of these molecular and also some other serological techniques is low and their specificity is not studied at all. This finding suggests that these more advanced tests need to be adapted to the specific setting in which they are used, i.e., in the tsetse fly and thus require further adequate designing to improve their performance.

6.4. Prospects and recommendations

African trypanosomosis can be fatal for both humans and animals. Tsetse flies function as the main biological vector of African trypanosomes. Trypanosomosis can be controlled by targeting either the parasite or the vector.

The parasite can be controlled by trypanocidal drugs or rearing of trypanotolerant breeds. However, targeting the parasite through trypanocidal drugs is no longer a viable option due to the presence of drug resistance; trypanocidal drugs are therefore not useful anymore in blocking the transmission, although it has been demonstrated that animals still benefit from receiving trypanocidal drugs, even though the animal is not completely cleared from the parasite, and thus remains a source of infection to the surrounding naïve animals. On the other hand, the rearing of trypanotolerant breeds is not economically rewarding in terms of meat and milk production.

Therefore, a more long lasting and economically viable strategy is to reduce or eradicate the vector. Wild animal removal and bush clearing were used in the past. Wild animal removal starves tsetse flies as it deprives food source whereas bush clearing destroys tsetse habitats. Both approaches are now unacceptable from an ecological point of view. This vector control intervention was therefore replaced by the use of toxic and persistent insecticides that directly kill the tsetse flies, which has been widely replaced now by non-persistent insecticides due to environmental concerns. The insecticides were –and are- sprayed in tsetse infested regions by aircraft as a blanket application, on the ground by spraying or are more recently applied in a more discriminative way assisted by tsetse fly density surveys. Additionally, odour-baited insecticide impregnated traps or targets are evolved to minimize the residual side effects of the persistent insecticides on untargeted pest and environment. Although traps and targets are useful, they are subjected to theft, require regular periodic replacement, repair, replenishment of attractants, and are sometimes difficult to effectively implement due to accessibility problems. When traps or targets are not properly maintained, flies reinvade previously cleared regions quickly from surrounding not cleared regions. Another use of insecticides is through spraying of livestock (pour-on) which kills tsetse flies that land on animals for feeding. Its large-scale use could subject animals in enzootic instability, particularly new-borns, to tick-borne diseases. Overall, therefore, pour-on and targets are proved to be inefficient to eliminate tsetse flies and also economically not feasible to apply in a large area on a global scale. As a result, sequential aerial spraying (SAT) is currently introduced and is applied over a wider area in large-scale

strategies using very low dosages of non-residual (non-persistent) insecticides such as endosulfan, synthetic pyrethroid insecticide (deltamethrin) or a cocktail of both. SAT currently utilizes aircrafts that are guided by a GPS-GIS navigation guidance system and spray management equipment to accurately release the correct dose to the correct place. The absence of resistance to the insecticides in tsetse flies despite insecticides being in use for a long time, makes SAT a viable and preferred approach. SAT may not eliminate the tsetse fly populations completely, not capable of eradicating the remaining tsetse flies when the tsetse density gets low. This thus poses a risk of a new build-up of the tsetse fly population after SAT is halted. To overcome this problem, SAT can be supplemented with the sterile male technique (SIT) to reduce the already suppressed tsetse fly population to an even lower density. This is done via the release of irradiated sterile males to tsetse suppressed areas using GPS-GIS assisted aircraft. The re-invasion from an uncontrolled area to a controlled area between the successive SAT spray operations could be a problem when applying the combination of the SAT and SIT strategy. The re-invasion problem during large-scale SAT or SIT campaigns could be prevented by (i) integration and deployment of targets, traps, insecticide-treated cattle or some combination of the three in cleared areas to prevent re-invasion, (ii) tackling the problem at regional level via collaboration of neighbouring counties, (ii) treating whole regions that are physically disconnected from regions with abundant tsetse fly populations. Once an entire region is cleared of tsetse flies, it remains important to continue surveillance with sensitive diagnostic tools. Such a tool is the anti-tsetse saliva ELISA as it enables the field workers to assess whether cattle has been challenged by tsetse flies in the last few months. If so, immediate action can be undertaken to avoid that a new tsetse fly population establishes itself.

A recent worrisome finding is the mechanical transmission of trypanosomes by other biting flies such as *Tabanus* and *Stomoxys*. This needs further investigation. The frequency and importance of such mechanical transmissions needs to be assessed, and an important unanswered question is whether mechanical transmission alone is sufficient to establish the parasite in the cattle population. .

With properly consorted and continued actions based on SIT and SAT and supplemented by other tools such as traps, targets and pour-ons, the tsetse fly population can surely be eradicated or reduced to such low numbers that the trypanosomes can no longer survive.

6.5. References

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Summary

Summary

Tsetse fly-transmitted African trypanosomosis is caused by three groups of trypanosome species which infect a range of hosts. Such infections lead to varying degrees of parasitemia, anemia, tissue damage and possibly death of the infected host depending on the trypanosome. Trypanosomosis has a significant impact on the economy and food production in Africa. Control interventions targeting the vector and/or the parasite are crucial in eradicating or decreasing the incidence of this devastating disease, thus, have a marked impact on the economy and food production in the region. A key input for guiding tsetse fly and trypanosomosis control is a good insight in the spatio-temporal distribution of tsetse flies and trypanosomosis, based on well-designed sampling and adequate detection, monitoring and surveillance tools.

This dissertation contains both a literature review and own experimental work. The general introductory part in **Chapter 1** presents an overview of trypanosome–tsetse–host interactions and reviews the epidemiology and control of the parasite and the vector. The diverse trypanosome species and the varying effects they have on a range of potential hosts are discussed. The tsetse fly is the most important vector of trypanosomes. Thus, the population structure and the reproduction cycle of tsetse flies is reviewed along with the diurnal activity, mobility, dispersal and host finding/locating behavior. In the review attention is also given to the host preference of the tsetse fly, the role of tsetse fly saliva in tsetse feeding and trypanosome transmission as well as to the trypanosome-tsetse fly interaction and factors limiting tsetse fly infections. Detection, monitoring and surveillance tools that demarcate the distribution and abundance of tsetse fly and trypanosomosis are reviewed as these tools are essential in following up the tsetse fly population and incidence in cattle. As indicated in the review, the current trypanosomosis control options have shifted to tsetse fly control as drugs and vaccine development against the parasite have failed due to the emergence of drug resistance and immune evasion of the parasite, respectively.

Chapter 2 introduces the rationale and objectives of this thesis. The main objective of this thesis was to quantify the abundance of tsetse flies using traditional traps and using anti-tsetse saliva ELISA test and the trypanosome infection prevalence in the tsetse flies and cattle in Ethiopia.

The last countrywide survey of tsetse fly and trypanosomosis abundance in Ethiopia dates back to the late 1970s. More recent information is restricted to particular locations. Information on vector and parasite distribution as well as on risk factors for trypanosomosis among the domestic hosts are of paramount importance to control the vector and the disease with the ultimate goal of achieving elimination. **Chapter 3** presents the results of a recent countrywide survey of tsetse fly and trypanosomosis abundance in Ethiopia. Tsetse flies and trypanosomosis are still abundant

and widely distributed in Ethiopia despite decades of control efforts. Of the currently widespread distribution of four tsetse fly species (*G. pallidipes*, *G .m. submorsitans*, *G. fuscipes* and *G. tachnoides*) in Ethiopia, only one tsetse species has been observed in Amhara region (*G. tachnoides*) and Benishangul-Gumuz region (*G. m. submorsitans*) whilst more than one tsetse fly species have been observed in Oromia region. The overall prevalence of cattle trypanosomosis over the 82 selected PAs is 9.6%; with 0% in 12 PAs, 1-5% in 17 PAs, 5-10% in 28 PAs, 11-15% in 15 PAs and 15-31% in 8 PAs indicating substantial variation between the PAs. With respect to the presence of the different trypanosome species, 76% was *T. congolense*, 18% *T. vivax*, 3.6% *T. brucei* and 2.4% mixed infections of *T. congolense* and *T. vivax*. The current survey highlights regions where tsetse flies and trypanosomosis are most abundant and where frequent transmission of the trypanosomes to livestock occurs. Accordingly, spatial factors such as low altitude (<1200m), major drainage system, local factors at peasant association level such as the presence of game reserves and land utilization/encroachment determine the presence of major breeding habitats for tsetse flies and thus trypanosome transmission. Surprisingly, no significant relationship is observed between tsetse fly density and prevalence of cattle trypanosomosis which warrants further study.

Tsetse fly saliva proteins play a key role in tsetse fly feeding and trypanosome transmission but they are also highly immunogenic. In **Chapter 4**, new ELISA test based on tsetse fly saliva proteins is described that is capable to quantify the contact of the host with the tsetse fly through tsetse fly bites. Total tsetse saliva protein and a recombinant version of Tsal1 (rTsal1) are evaluated in an indirect ELISA to quantify the contact with total *Glossina morsitans morsitans* saliva, and thus the tsetse fly bite exposure. Mice and pigs were experimentally exposed to different *G. m. morsitans* exposure regimens, followed by a long-term follow-up of the specific antibody responses against total tsetse fly saliva and rTsal1. The study revealed that both total tsetse saliva and recombinant Tsal1 protein are sensitive immunological probes to detect contact with tsetse flies. The new serological test at hand is a sensitive indicator that can differentiate regimens of tsetse fly bites (various degree of exposure of animals) and that can also detect tsetse fly bite after a prolonged period without exposure. This serological test could thus be used in a complementary way next to entomological surveys in tsetse fly prevalence study, or to monitor the impact of vector control programs and to detect re-invasion of areas previously cleared for tsetse flies.

The proportion of tsetse flies infected with trypanosomes is a function of the infection prevalence and parasitemia in the hosts. Conversely, trypanosome infection prevalence in the tsetse fly is one

of the fundamental parameters of AAT risk analysis in estimating the risk of an individual animal to acquire a trypanosome infection. However, the prevalence of trypanosome infections in the tsetse flies is often a neglected parameter probably due to the intensive labor required for its evaluation. A systematic review (**Chapter 5**) has been conducted on trypanosome infection prevalence of tsetse flies either collected in the field or in laboratory controlled conditions, based on all published information available since 1950s. The overall prevalence of trypanosome infection in tsetse fly populations was 10.3% and 31.0% for the field survey data and laboratory experiment data, respectively. Both spatial (country) and temporal (year of publication) variation are noticed in trypanosome infection prevalence of field collected tsetse flies. It is essential for targeting interventions to identify areas and time periods with high prevalence of trypanosome infection in the tsetse flies. Dissection is still widely used as a diagnostic method although molecular and serological techniques are gradually finding their way as well. Unfortunately, the sensitivity and specificity of currently available molecular and serological techniques work apparently suboptimal for tsetse fly samples as they are developed and optimized for trypanosome detection in the host. This warrants further investigation before they can be employed on a routine basis to determine the presence of trypanosomes in the tsetse fly. Comparing several tests on the same specimen panel would allow more accurate comparisons of the sensitivity and specificity. Integrating the prevalence of trypanosome infection in the tsetse fly in monitoring programs would allow a more precise evaluation of the risk of being infected in a particular region. In this regard, the broader perspective of the findings are discussed in **Chapter 6** along with the limitations and avenues for future research.

Samenvatting

Samenvatting

De Afrikaanse slaapziekte die door tsetse vliegen wordt overgebracht, wordt veroorzaakt door drie groepen van species van trypanosomen, die voorkomen in verschillende gastheren. Dergelijke trypanosomeninfecties leiden tot verschillende niveaus van parasitemie, anemie, veroorzaken minder of meer weefselschade en kunnen eventueel ook de dood tot gevolg hebben afhankelijk van het type van trypanosoom. De Afrikaanse slaapziekte heeft een grote impact op de economie en op de voedselproductie in Afrika. Interventies die er op gericht zijn de vector en/of de parasiet te controleren zijn essentieel in het elimineren of het terugdringen van deze belangrijke ziekte; op die manier hebben die interventies een belangrijke input in het versterken van de economie en de voedselproductie. Om de controle-interventies tegen de vector en de parasiet zo succesvol mogelijk te maken is het essentieel om een goed inzicht te hebben in het voorkomen van tsetse vliegen en de Afrikaanse slaapziekte in tijd en ruimte, en dergelijke informatie kan verzameld worden door het nemen van goed geplande steekproeven waarbij gebruik gemaakt wordt van performante diagnostische testen.

Dit doctoraal proefschrift bevat zowel een literatuurstudie als eigen wetenschappelijk experimenteel onderzoek. De algemene inleiding in **Hoofdstuk 1** stelt een overzicht voor van de interacties tussen de trypanosoom, de parasiet en de vector, en de epidemiologie en de controle van parasiet en vector. De verschillende trypanosoma species en het effect dat ze hebben op verschillende gastheren worden besproken. De tsetse vlieg is de belangrijkste vector van trypanosomen. Vandaar worden een aantal belangrijke aspecten in de levenscyclus van de tsetse vlieg besproken: de populatiestructuur, de reproductie, de activiteiten tijdens de dag, de mobiliteit, de verspreiding, en verder hoe tsetse vliegen hun gastheer vinden en zich vervolgens voeden. Tsetse vliegen verschillen ook in preferentie voor bepaalde gastheren. Het speeksel van de tsetse vlieg speelt verder ook een cruciale rol in de voeding en de transmissie van trypanosomen. Er zijn tenslotte een aantal factoren die bemoeilijken dat de tsetse vlieg geïnfecteerd wordt met trypanosomen. In het hoofdstuk worden ook verschillende diagnostische testen besproken die toelaten de aanwezigheid van tsetse vliegen en Afrikaanse slaapziekte te kwantificeren omdat ze essentieel zijn in het opvolgen van de ziekte in specifieke regios. Zoals aangegeven in het hoofdstuk zijn controletechnieken vooral gebaseerd op de vector omdat medicijnen niet efficiënt meer zijn door het voorkomen van resistentie van de parasiet en er nog steeds geen vaccin ontwikkeld is omdat de parasiet er in slaagt om de immuunreactie van de gastheer te ontlopen.

Hoofdstuk 2 introduceert de doelstellingen van het doctoraatsproefschrift. Het hoofddoel bestaat erin de aanwezigheid van tsetse vliegen in Ethiopië te kwantificeren door gebruik te maken van

tranditionele vallen en daarnaast ook van anti tsetse vliegen speeksel ELISA testen. Ook de prevalentie van trypanosomeninfectie in de tsetse vlieg in Afrika werd geëvalueerd.

De laatste grote survey van tsetse vliegen en Afrikaanse slaapziekte die heel Ethiopië overschouwde werd uitgevoerd in de late jaren ’70. Meer recente informatie is beperkt tot een specifiek aantal locaties. Toch is de informatie over de verdeling van de vector en de parasiet, tesamen met eventuele risicofactoren, in Ethiopië essentieel om de vector en de ziekte te bestrijden met het uiteindelijke doel om ze uit te roeien. In **Hoofdstuk 3** worden de resultaten voorgesteld van een recente survey van tsetse vliegen en Afrikaanse slaapziekte over heel Ethiopië. Tsetse vliegen komen nog steeds veelvuldig en verspreid voor over grote delen van Ethiopië, ondanks alle interventies die de laatste tientallen jaren zijn uitgevoerd. Vier species van tsetse vliegen komen wijdverspreid voor in Ethiopië: *G. pallidipes*, *G .m. submorsitans*, *G. fuscipes* en *G. tachnoides*. In de Amhara regio wordt slechts één species, *G. tachnoides*, aangetroffen behalve op een beperkt aantal locaties ook *G. pallidipes*, en in de Benishangul-Gumuz regio wordt enkel *G. m. submorsitans* gevonden. In de Oromia regio komt typisch op dezelfde plaats meer dan één species voor. De globale prevalentie van Afrikaanse slaapziekte bij vee in de 82 geselecteerde boerenassociaties (BA) is 9.6%; met 0% in 12 BAs, 1-5% in 17 BAs, 5-10% in 28 BAs, 11-15% in 15 BAs en 15-31% in 8 BAs wat aangeeft dat er substantiële variatie bestaat tussen de boerenassociaties. Wat het het voorkomen van de verschillende trypanosoma species aangaat, was 76% *T. congolense*, 18% *T. vivax*, 3.6% *T. brucei* en 2.4% gemengde infecties van *T. congolense* en *T. vivax*. Deze nieuwe survey geeft aan in welke regios de meeste tsetse vliegen en trypanosomen voorkomen en waar er veelvuldig transmissie is tussen de vlieg en de gastheer. Regios met hoge transmissie worden vooral teruggevonden op lagere hoogte (<1200m) op plaatsen waar voldoende water aanwezig is, en ook lokale factoren op niveau van de boerenassociaties zoals de aanwezigheid van wildparken en intensief landgebruik kunnen bijdragen tot hogere incidenties van Afrikaanse slaapziekte in vee. Er werd geen verband gevonden tussen de dichtheid van de tsetse vliegen en het voorkomen van Afrikaanse slaapziekte bij het vee. Dit onverwachte resultaat dient verder onderzocht te worden.

Speeksel proteïnen van de tsetse vlieg spelen een belangrijke rol bij het voeden en bij het overdragen van de trypanosomen, maar zijn eveneens immunogeen. In **Hoofdstuk 4** worden nieuwe ELISA testen beschreven gebaseerd op tsetse vliegen speeksel proteïnen die toelaten om het contact tussen de gastheer en de tsetse vlieg te kwantificeren in termen van het aantal tsetse vliegen beten. Zowel totale tsetse speeksel proteïne en een recombinante versie van Tsal1 (rTsal1) werden geëevalueerd met een indirecte ELISA techniek om het contact met *Glossina*

morsitans morsitans speeksel te kwantificeren, wat in principe overeenkomt met hoe frekwent de gastheer door een tsetse vlieg gestoken wordt. In een experiment werden muizen en varkens blootgesteld aan *G. m. morsitans* e, waarbij zowel het aantal tsetse vliegen per blootstelling en het aantal blootstellingen werd gevarieerd. Vervolgens werden de dieren lange tijd opgevolgd voor specifieke antibody respons tegen het totale tsetse vlieg speeksel en rTsal1. Uit het experiment bleek dat zowel het totale tsetse vlieg speeksel als het recombinante Tsal1 kunnen gebruikt worden. De nieuwe serologische test is een sensitieve indicator waarmee verschillende graden van blootstelling kunnen worden onderscheiden en bovendien kan een nieuwe beet gedetecteerd worden na een langere periode zonder blootstelling. De test kan dus gebruikt worden op een complementaire manier naast een entomologische survey in een tsetse vlieg prevalentie studie, of om de impact van een vector controle programma op te volgen of ook nog om zeer snel een re-invasie vast te stellen in gebieden die voorheen geklaard waren van tsetse vliegen.

De proportie tsetse vliegen die geïnfecteerd zijn met trypanosomen is een functie van de infectieprevalentie en parasitemie in de gastheer. Anderzijds is de prevalentie van trypanosoma infecties in de tsetse vlieg een fundamentele parameter om het risico in the schatten dat een individueel dier zal geïnfecteerd worden met trypanosomen. De prevalentie van trypanosoma infecties in de tsetse vlieg wordt vaak genegeerd waarschijnlijk omdat het arbeidsintensief is om het te kwantificeren. Een systematisch overzicht (**Hoofdstuk 5**) over de prevalentie van trypanosoom infectie in de tsetse vlieg werd samengesteld zowel voor veldgegevens als voor gegevens die in gecontroleerde laboratoriumcondities werden gegenereerd gebaseerd op alle gepubliceerde informatie sinds 1950. De globale prevalentie van trypanosoom infectie in de tsetse vliegen populatie was 10.3% en 31.0% voor de veldstudies en de laboratorium experimenten respectievelijk. Zowel spatiale (land) als temporele (jaar van publicatie) variatie waren aanwezig in de prevalentie van de trypanosoom infectie van de tsetse vliegen uit de veldstudies. Het is essentieel om regios en periodes te identificeren met hoge prevalentie van trypanosoom infectie in de tsetse vliegen om op de juiste plaats en tijd te kunnen interveniëren. De dissectie method is nog steeds de meest gebruikte diagnostische methode maar stilaan doen ook moleculaire en serologische technieken hun intrede. De sensitiviteit en specificiteit van deze moleculaire en serologische technieken zijn op dit moment nog te laag of ongekend om ze op een grote schaal bruikbaar te maken. Dit heeft waarschijnlijk te maken met het feit dat de testen ontwikkeld zijn om trypansomen in het bloed van de gastheer te detecteren. De staalvoorbereiding voor het detecteren van trypanosomen in de tsetse vlieg zal bijgevolg

geoptimaliseerd moeten worden. Verschillende diagnostische testen zouden moeten vergeleken worden op dezelfde set van positieve en negatieve stalen zodat de sensitiviteit en specificiteit van de verschillende testen kan vergeleken worden. Het integreren van de prevalentie van trypanosoom infectie in de tsetse vlieg in monitoring programmas zou de predictie van infectie in een bepaald gebied en tijdspanne substantieel kunnen verbeteren, en zo ook aangeven waar interventie nodig is.

Tenslotte wordt de meer algemene conclusies van het geheel van het werk besproken in **Hoofdstuk 6**.

Curriculum vitae

Curriculum vitae

Reta Duguma Abdi was born in 1975 in rural areas of Wollega near Shambu and grown having fun with livestock, drinking their fresh milk. He followed his primary education at Haroshote junior school, elementary at Shambu and high school partly at Shambu and at Jiren, Jimma. He joined Addis Ababa University and graduated from Bishoftu campus in 2001 as a veterinarian (DVM). He was employed at Bako Agricultural Research Center in 2002, then joined Bishoftu Agricultural Research Center in 2003 and later employed at the College of Veterinary Medicine and Agriculture of the Addis Ababa University (AAU) in 2006. In 2007 he got a Scholarship for his Masters Study in Belgium and graduated in 2008 whereby he did his master's thesis on "Monitoring *Theileria annulata* attenuation for vaccine using real time-PCR quantification for gene expression levels of some parasite and bovine leukocyte proteins in different cell cultures passages" at Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium. In 2010 he won an individual PhD scholarship competition grant and joined a PhD study at Gent University and Prince Leopold Institute of Tropical Medicine, Belgium.

Being motivated in research, Reta has won a number of research grants to undertake applied research: *(i)* VLIR South Initiative (SI) from Belgium on novel tsetse fly monitoring tools, *(ii)* AAU thematic research grant on exploration of epidemiology, ecology & drug resistance of *Salmonella* & *Staphylococcus*, *(iii)* Ministry of Science and Technology of Ethiopia on thermostable Newcastle disease vaccine trial, *(iv)* World Bank and Canadian International Development Agency channelled via Ethiopian Agricultural Research Institute (EIAR) for National Agricultural Research Fund on improving Newcastle and Marek's disease vaccine delivery system, *(v)* World Bank-Agricultural Research and Training Project channelled via EIAR for Agricultural Research on developing a chicken production model to reduce poverty in urban areas. He gave speech to Ethiopian radio and Television interview in 2006 and in 2011 for his distinctive research findings.

He has participated in a number of committees in the College of Veterinary Medicine and Agriculture and AAU, in professional associations as well as on (inter)national scientific workshops. He got a number of trainings on advanced data analysis (linear, logistic regression & survival analysis), real-time PCR, ELISA, SDS-PAGE electrophoresis and Western blot analysis.

He published more than 20 articles on international journals, advised more than 20 MSc and over 25 DVM students. Currently, he is an Associate Professor at AAU, married and a father of a son and a daughter.

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